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A Review of Progress in the Study of the Genetics of Spontaneous Tumor Incidence

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Before discussing the various results obtained in experiments on the genetics of spontaneous tumors, it may be well to point out certain factors which determine to a considerable degree the extent to which the data derived from various sources are suitable for analysis.

Spontaneous tumors are found chiefly in the middle- or advanced-age groups of men and of animals. Those occurring earlier are apt to belong to more or less restricted or specialized types, the genetics of which might well be expected to differ from the common varieties. This fact does not make the earlier tumors any less interesting but serves as a reason for caution in attempting any general analysis or in accepting any broad conclusions based on them alone.

Very naturally popular interest is apt to focus on the degree to which heredity plays a role in the incidence of spontaneous tumors in man. It should, however, be borne in mind that the fact that a tendency to form tumors is inherited is not in itself a guarantee of the practical importance of that process. How the tendency is inherited is far more important than the bare demonstration that heredity plays a part in its etiology.

The mere fact of inheritance of a tendency or tendencies to form different types of tumors has been fairly satisfactorily demonstrated by various sorts of statistical analysis both of grouped data and of individual pedigrees. Among the latter type of material, studies on tissues have played an important and interesting part.

It is, however, unlikely that any such studies can give us more information about the "how" of the process of inheritance. The data themselves lack the fundamental controls necessary in order to evaluate quantitatively any observed grouping of familial incidence of cancer. This fact should be especially remembered when the question of human material is under discussion.

A somewhat similar situation exists in animal material. Early studies of cancer incidence in successive generations of laboratory rodents (particularly mice) were made by using the "pedigree" method of collecting and tabulating data. The inci-

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dence of cancer in a given family was mapped by individual matings, and the genetic nature of its reappearance was estimated and analyzed as the succeeding generations gave increasing data. Although such data can be suggestive, the uncontrolled residue of genetic and nongenetic influences which may affect the appearance of cancer is an unavoidable factor in decreasing the definiteness and predictability of results.

The fact is that simplification of the situation by the reduction of variables is a prerequisite to the laying of a foundation for accurate work. The surest way in which this can be accomplished is by intensive inbreeding for a sufficient number of generations to produce a close approximation to genetic uniformity. This procedure, accompaned by due attention to controlling the factors of age, sex, reproductive activity, diet, etc., will be time-consuming but will be the most practical guarantee that cause-and-effect relationships can be detected and analyzed.

With this introductory statement, I shall review briefly the development of genetic research on cancer incidence in human and animal material.

HUMAN MATERIAL

One of the earlier textbooks on genetics and eugenics written after the rediscovery of Mendelism was that of Davenport (*31*), in 1911. Davenport devoted two pages to a discussion of cancer in relation to heredity and concluded that there is "basis for the conclusion that there is a family liability to cancer" and that there is further, "a specificity of the disease in each particular family."

Macklin (*59–63*) has repeatedly emphasized the familial incidence of similar types of tumors as a strong indication that genetic factors were involved.

There is no doubt that the "greater than chance" incidence of certain types of tumors in a given family or in members of pairs of similar twins proves that individuals of relatively similar biological origin tend to reproduce conditions conducive to the formation of similar types of neoplasm. This can be shown by studies of twins (McFarland (*58*), Macklin (*63*)), by pedigree charts (Warthin (*78, 79*), Davenport (*31*)), or by general statistical comparisons of tumor incidence in parents and offspring or among sibs (Little (*46*), Wassink (*80*)).

The fact is, however, that the existence of such increased incidence of cancer does not in itself prove or demonstrate the effect of heredity in the chromosomal sense.

In the case of similar twins, for example, we know that the two individuals were at an early stage of development actually one. When, therefore, both members of a pair of twins develop along similar lines and show similar disturbances of growth at comparable ages, we know that their course of development was largely predetermined before the time of their separation.

We do not, however, know whether this predetermination came from the chromosomes, or the cytoplasm, or some other cellular element, or combination of such. We do not know whether the similarity depends upon a process of true heredity or on direct transmission of some influence or influences.

All that such studies can accomplish, therefore, is to make us wonder why in the past 40 years, since genetics began to be an experimental science, there has been so little interest in setting up a center at which accurate human data based on direct observation can be collected for an indefinite period in the future.

There are certainly too many instances

of the incidence of cases of similar types of tumor in biologically restricted groups to be explicable on chance alone. The effort to determine the factors that contribute to this situation would have been worth making and still is in need of serious consideration.

How far we are from the desired degree of development of such a program is shown by the fact that in his 1930 edition of Genetics and Eugenics, Castle (*27*) made no mention whatever of cancer in the index nor did he include it among the human diseases even remotely affected by heredity. Holmes (*38*), in 1936, mentioned cancer briefly but with no more definite knowledge than that possessed by Davenport 25 years earlier.

To sum up the situation, therefore, we may say that for more than half a century there has been accumulating from various types of material clear evidence that biological and constitutional similarity affect the incidence of cancer in humans.

The various methods of analysis have been interesting, clever, and suggestive, but they have not proved to be an adequate substitute for direct observation nor can they ever be.

There is no valid evidence against the presence of genetic influence on incidence of human cancer, and there are some preliminary facts in favor of it.

There exists at present, however, no basis whatever for the application of genetic principles to the problem of cancer incidence in the case of any individual human being.

ANIMAL MATERIAL

Among laboratory animals the situation is quite different. Although there are no established inbred cancer-producing strains of dogs, cats, or guinea pigs with which to work, a beginning has been made with rabbits (Greene (*34–38*)) and with rats (Bagg and Jacksen (*5*)). As time goes on, the data derived from these forms will increase and become more valuable.

At present, however, the animal material which exists under most controlled genetic conditions and which, therefore, provides the backbone of experimental cancer research, is mice.

With these animals, the methods of experimental genetics have been used with two major objectives in mind. The first of these is to discover what we can about the actual nature of the genetics of cancer in mice. The second is to use the process of continued and intensive inbreeding to provide as nearly as possible homogeneous genetic material for use in investigation of all sorts of physical, chemical, physiological, immunological, and other problems of experimental medicine.

We may profitably consider a brief review of the main steps of progress in this field.

PROGRESS OF GENETIC STUDIES

The earliest genetic experiments with spontaneous cancer in mice were performed by Tyzzer (*75–77*), Bashford, J. A. Murray (*64*), Haaland (*39*), and Lathrop and Loeb (*44, 45*).

In some cases the number of animals studied was not so large as it might well have been. In others, microscopic diagnosis of subcutaneous nodules was not completed. In all the degree of knowledge concerning the remote ancestors of the experimental mice was not great. In no case had genetic uniformity of the strains used previously been attained by the required amount of close inbreeding.

This also handicapped the later work of Slye and of Lathrop and Loeb.

In spite of these facts, however, several conclusions of considerable importance were reached:

(1) Cancer occurred more frequently in some strains than in others.

(2) These differences were usually maintained in succeeding generations.

(3) There was evidence of some specificity of the site of tumors which could be fixed to a considerable degree within a strain.

(4) There tended to be a characteristic mean age at which tumors of a given site occurred within each strain.

Into the establishment of these conclusions went an immense amount of experimental data and a noteworthy degree of patient observation, especially by Slye (*70*, *71*). Her work went as far as it was humanly possible to go by the pedigree method of analysis.

This type of analysis, however, has to depend by its very nature upon the ex post facto classification of strains as modified by the rate of incidence and types of tumors observed in individual families during the process of breeding.

As these various data were being collected and analyzed, it became clear that the genetic situation was complex and difficult of explanation.

This was not surprising for experiments of Wright (*81*, *82*) and others had shown that the genetics of various morphological abnormalities in rodents was complicated by the internal environment of the individual as well as by multiple genes.

For these reasons it will be well to group certain types of tumors and to consider separately these groups as follows.

(1) Mammary epithelial tumors.
(2) Epithelial pulmonary tumors.
(3) Nonepithelial tumors.
(4) Leukemias.

MAMMARY EPITHELIAL TUMORS

The first critical and extensive evidence on the genetic behavior of mammary epithelial tumors was obtained by Lynch who showed that tumors occurred in the F_1 generation of a cross between low-tumor and high-tumor strains.

This fact indicated that the tendency to form such tumors was possibly partly dominant and conflicted with the theory then being advanced by Slye that the tendency to form all types of cancer was brought about by a Mendelian recessive gene.

The next step was a communication by the staff of the Roscoe B. Jackson Memorial Laboratory (*41*), which was almost immediately followed by one by Korteweg (*43*), showing independently that reciprocal crosses between high- and low-mammary-tumor strains gave strikingly and unexpectedly different results. Not only was this true of the F_1 generation, but the difference persisted in the F_2 generation with very little change.

Tables taken from a slightly later paper by Murray and Little (*66*) gave the following results in virgin females which lived as long or longer than the first animal of their generation to form a tumor.

Parent	*Percentage of tumor incidence*
High tumor ♀ × low tumor ♂ F_1 . .	39.82
Low tumor ♀ × high tumor ♂ F_1 . .	6.06
High tumor ♀ × low tumor ♂ F_2 . .	35.54
Low tumor ♀ × high tumor ♂ F_2 . .	5.96

Since the chromosomal composition of the females of the two reciprocal F_1 generations was the same and their tumor incidence was strikingly different, it was evident that an extrachromosomal influence of some sort was active.

The two F_2 generations could also properly be considered as similar populations in so far as distribution of genes was concerned, and the difference which was found there provided additional evidence that the chromosomes alone could not explain the observed facts.

Later work by Bittner (*20–24*) has tended to show that the incidence of breast tumors may be influenced by genes as well as by other agents. The exact degree of this

influence is, however, undetermined, and it is certain that it plays a minor role in determining the incidence of this type of tumor.

Bittner believes that whatever gene influence exists is more dominant than recessive in its action.

EPITHELIAL PULMONARY TUMORS

We may at the outset note that there is no evidence of any reciprocal-cross difference as far as the incidence of pulmonary tumors is concerned so that the situation is not so complex as that of breast-tumor formation.

Lynch (*52–56*) published the first data on crosses between high-and low-pulmonary-tumor strains. Although her material had not been rendered genetically homogeneous by a long-continued process of inbreeding, it gave suggestive and valuable results. The tendency to form tumors of the lung gave clear evidence of being at least partially dominant.

The most conclusive evidence came in a somewhat later paper by Bittner, who obtained the following results: In 133 virgin females of "low" parent stock, the incidence of pulmonary tumors was 0.0 percent; in 221 virgin females of "high" parent stock, 90.0 percent; in 373 F_1 virgin females, 87.5 percent; and in 376 F_2 virgin females, 67.3 percent.

These figures are close to the theoretical expectation on the hypothesis that the tendency to form pulmonary tumors is a Mendelian dominant and that about 10 or 11 percent of those animals genetically "tumorous" fail for some reason to form a detectable neoplasm.

The presence of animals genetically of an abnormal type but somatically normal as far as macroscopic examination is concerned, is a well-known phenomenon so that the incidence of 10 or 11 percent of "normal overlaps" among what should be individuals with cancer of the lung is nothing exceptional.

Recent work by Heston [1] seems to indicate that there are modifying genes present in certain low pulmonary-tumor strains which influence the degree to which the tendency to form pulmonary tumors is dominant and which also affect the degree to which it expresses itself in segregation in the F_2 generation. This naturally complicates the situation.

There is also evidence from the work of Heston, Andervont (*1–4*), and others that there is a genetic basis (probably multiple factors) which influences the number of tumor nodules formed in the lungs of any one individual.

To summarize, we may say that the genetic influence in the incidence of epithelial tumors of the lung in mice has been established.

This influence and the way in which it affects rate of incidence and number of nodules appears to be that of a Mendelian dominant complicated in some crosses by the appearance of normal overlap and modifying genes.

NONEPITHELIAL TUMORS

Very obviously the heading, Nonepithelial Tumors, in itself, indicates that we have not yet obtained a sufficient mass of accurate data to enable us to separate the many different types of nonepithelial tumors in our analysis of the problem.

There are certain facts, however, which tend broadly to differentiate tumors of this type from epithelial tumors of the breast and lung. Among these are the age at which the two sorts occur. As a general thing nonepithelial tumors appear distinctly later than do those of epithelial origin.

[1] *Heston, W. E.: Unpublished data.*

A comparison of the mean ages in days at death of animals of various stocks and hybrids between them, with epithelial or with nonepithelial tumors, is given in table 1.

TABLE 1.—*Mean ages at death of mice of various stocks and hybrids between them, with epithelial and nonepithelial tumors*

Stock or generation	Age of mice with mammary tumors of epithelial origin	Age of mice with nonepithelial tumors	Excess in mean age of nonepithelial group
	Days	*Days*	*Days*
Jax C57	[1]	706	
Jax dba	433	[1]	
dBF_1	575	806	+231
BdF_1	711	808	+97
dBF_2	566	706	+140
BdF_2	623	704	+81

[1] Not sufficient tumors of this type to provide critical data.

Another difference is the comparative difficulty in localizing within a strain of mice the site at which nonepithelial tumors will appear. Similarly the types of tumors were found, by histopathological diagnosis, to be varied.

Table 2 gives the distribution of 121 nonepithelial tumors in a single inbred stock of mice (Jax C57 Black), and the classification of these same 121 tumors.

TABLE 2.—*Distribution of 121 nonepithelial tumors in Jax C57 Black mice, classified according to site* [1] *and kind*

Site of tumor	Distribution		Kind of tumor	Distribution	
	No.	*Pct.*		*No.*	*Pct.*
Spleen and nodes	75	62.0	Lymphoblastoma	73	60.0
Liver	31	25.6	Endothelioma	26	21.5
Uterus	4	3.3	Fibrosarcoma	8	6.6
Mammary line and branches	4	3.3	Lymphangioma	5	4.1
Subcutaneous (side or appendages)	3	2.5	Osteogenic sarcoma	3	2.5
Tail	1	.8	Hemangioma	3	2.5
Jaw	1	.8	Melanoma	1	.8
Eye	1	.8	Reticulum-cell sarcoma	1	.8
Intestine	1	.8	Undifferentiated	1	.8

[1] Data from Little, Murray, and Cloudman (*51*).

Various studies have shown no sign of extrachromosomal influence in these various types of tumor, although it must be admitted that really comprehensive and critical tests have not yet been made.

There is also no significant difference between the sexes as regards rate of incidence of nonepithelial tumors, although there is some indication that perhaps more carefully collected and more extensive data may show that in certain cases such a difference exists.

As yet there is no evidence that any simple Mendelian theory will explain the observed genetic facts in the incidence of these tumors. Very evidently dominance, if present, is so incomplete as to be difficult of determination. Since there also seems to be a large percentage of "nontumor" mice in inbred strains where tumors also occur and where great genetic homogeneity can be fairly expected, it is clear that we are faced with a very complex situation that requires much more patient and time-consuming research before we can expect to unravel it.

LEUKEMIA

MacDowell and his associates (*57*) have done a large amount of careful and important work on the genetic behavior of spontaneous leukemia in mice.

Up to the present time their work indicates:

(1) That there are some normal overlaps (nonleukemic mice) even in the most homogeneous genetically high tumor lines.

(2) That in crosses between high and low leukemic lines the F_1 hybrids show an incidence of leukemia intermediate between that of the two parent strains.

(3) That there is some evidence of extrachromosomal influence with a tendency for the incidence of leukemia in reciprocal F_1 generations to resemble that of the strain from which the mother is derived more than it does that of the father's strain. This influence is not, however, so clearly defined as is that which affects the incidence of breast tumors.

GENERAL FACTORS

There is some evidence that general factors such as coat color and hybridization may, in certain cases, influence the incidence of spontaneous tumors.

Two of these factors may be very briefly considered.

Coat Color

There are two recorded instances of data that show probable relationship between coat color and the incidence of spontaneous tumors.

One of these concerns yellow coat color and the incidence of epithelial tumors of the breast and also tumors of lipoid tissue (Little).

Yellow mice are peculiar in certain respects. They have never been observed in a homozygous condition. Yellow coat color is a Mendelian dominant over non-yellow. The individuals that would be homozygous yellows die in utero as abnormal and malformed embryos.

The surviving yellows, therefore, always carry non-yellows as a recessive. They frequently develop much more adipose tissue than their non-yellow sibs. Lipomas or liposarcomas have been found in yellow animals in significantly greater numbers than in non-yellow mice of the same genetic strain.

In a single cross reported by the writer, hybrids of yellow × non-yellow developed 44 nonepithelial and 9 lipoid tumors, the percentage of lipoid tumors being 20.4; whereas hybrids of non-yellows (same strains) developed 199 nonepithelial, and no lipoid tumors.

In the same material a difference in the incidence of epithelial mammary tumors in yellow and non-yellow mice was also noted (table 3).

This difference may be an indication of the altered physiology of the yellows which are usually distinctly less fertile than their non-yellow sibs.

TABLE 3.—*Incidence of epithelial mammary tumors in yellow and non-yellow mice*

Generation	Strain		With mammary tumors	
	Yellow	Non-yellow	Yellow	Non-yellow
	Number	*Number*	*Percent*	*Percent*
F_1	57	54	38.6	64.8
F_2	156	223	37.2	51.6
Total	213	277	37.5	54.2

The second coat-color difference to be noted is still of doubtful significance although it is suggestive and worthy of further study. This involves the gene for dilute pigmentation which is a Mendelian recessive to the normal intense coat color.

In tabulating the incidence of nonepithelial tumors among intense and dilute pigmented mice in two series of experiments, differences were also noted. There was a slight but consistent excess of tumors among the dilute mice (table 4).

TABLE 4.—*Incidence of nonepithelial tumors among intense and dilute pigmented mice*

Series	Intense			Dilute		
	Mice	Tumors		Mice	Tumors	
	Number	*Number*	*Percent*	*Number*	*Number*	*Percent*
1	732	105	11.4	236	46	16.3
2	264	13	4.6	115	10	8.0
Total	996	118	10.6	351	56	13.8

Hybridization

The second general factor that appears to have a definite effect upon the incidence of tumors is that of hybrid origin of the animals studied.

The clearest evidence of this is derived from a cross between two species of mice that give fertile hybrids.

One parent species is *Mus bactrianus*, a small, slow-growing, slow-maturing, and relatively long lived form.

The other is a tame strain (Jax C57 Black) derived from the ordinary wild house mouse *Mus musculus*. These are large, fast-growing, early-maturing, and slightly shorter lived in comparison.

The incidence of nonepithelial tumors in the two parent strains and their F_1 hybrids is as follows: Of 159 *Mus bactrianus*, none developed tumors; whereas of 877 *M. musculus* (Jax C57), 116 (13.2 percent) developed tumors; of the 121 F_1 hybrids, 48 (39.7 percent) developed them.

There are also distinctly more hybrids which form multiple primary tumors than there are animals of this type in the parent strains. The percentage of tumor-bearing mice with multiple growths is as follows: *Mus bactrianus*, 0.0; Jax C57, 7.9; and F_1 hybrids, 23.6.

It is evident, therefore, that animals which have in their physiological make-up widely divergent tendencies as to rate and extent of growth may be apt to show growth abnormalities and disturbances in advanced age.

The study of the genetics of spontaneous tumor formation in mice has thus reached a point where some of the factors that contribute to the total process are being isolated by the use of inbred strains.

Already clear differences in the method of inheritance of the chief types of tumors have been recognized and established. A preliminary framework for the discovery of new facts has been built. Means of separating genetic and nongenetic, chromosomal and extrachromosomal influences have been devised. Definite appreciation of the physiological factors that may affect tumor incidence has been developed.

The future will need opportunities for uninterrupted work and patience and skill in its execution. The outlook, however, is distinctly encouraging.

BIBLIOGRAPHY

(*1*) ANDERVONT, H. B.: Pulmonary tumors in mice; further studies on influence of heredity upon spontaneous and induced lung tumors. Pub. Health Rep., 53: 232–237 (1938).

(*2*) ANDERVONT, H. B., and MCELENEY, W. J.: Incidence of spontaneous tumors in colony of strain C_3H mice. Pub. Health Rep., 52: 772–780 (1937).

(*3*) ANDERVONT, H. B., and MCELENEY, W. J.: Influence of nonbreeding and foster nursing upon occurrence of spontaneous breast tumors in strain C_3H mice. Pub. Health Rep., 53: 777–783 (1938).

(*4*) ANDERVONT, H. B., and MCELENEY, W. J.: Influence of foster nursing upon incidence of spontaneous breast cancer in strain C_3H mice. Pub. Health Rep., 54: 1597–1603 (1939).

(*5*) BAGG, H. J., and JACKSEN, J.: Value of "functional test" in selecting material for genetic study of mammary tumors in mice and rats. Am. J. Cancer, 30: 539–548 (1937).

(*6*) BASHFORD, E. F., and MURRAY, J. A.: The incidence of cancer in mice of known age. Proc. Roy. Soc., London, s. B, 81: 310–313 (1909).

(*7*) BITTNER, J. J.: Breeding behavior and tumor incidence of inbred albino strains of mice. Am. J. Cancer, 25: 113–121 (1935).

(*8*) BITTNER, J. J.: Breeding behavior and tumor incidence of black agouti stock of mice. Am. J. Cancer, 25: 614–620 (1935).

(*9*) ———: Differences observed in tumor incidence of albino strain of mice following change in diet. Am. J. Cancer, 25: 791–796 (1935).

(*10*) ———: Tumor incidence in reciprocal F_1 hybrid mice —A × D high tumor stocks. Proc. Soc. Exper. Biol. & Med., 34: 42–48 (1936).

(*11*) ———: Spontaneous incidence of lung tumors in relation to incidence of mammary tumors in inbred strain of albino mice (strain A); preliminary report. Am. J. Cancer, 27: 519–524 (1936).

(*12*) ———: Some possible effects of nursing on mammary gland tumor incidence in mice. Science, 84: 162 (1936).

(*13*) ———: Spontaneous tumor incidence in mice; "Z" stock × "I" stock. J. Hered., 27: 391–393 (1936).

(*14*) ———: Mammary tumors in mice in relation to nursing. Am. J. Cancer, 30: 530–538 (1937).

(*15*) ———; Some possible effects of nursing on mammary gland tumor in mice. Am. J. Clin. Path., 7: 430–435 (1937).

(*16*) ———: Breast cancer and mother's milk; relation of nursing to theory of extrachromosomal causation of breast cancer

in mice. Preliminary report. J. Hered., 28: 363–365 (1937).
(17) BITTNER, J. J.: Genetics of cancer in mice. Quart. Rev. Biol., 13: 51–64 (1938).
(18) ———: Spontaneous lung carcinoma in mice. Pub. Health Rep., 53: 2197–2202 (1938).
(19) ———: Relation of nursing to extra-chromosomal theory of breast cancer in mice. Am. J. Cancer, 35: 90–97 (1939).
(20) ———: Breast and lung carcinoma in "A" stock mice. Pub. Health Rep., 54: 380–392 (1939).
(21) ———: Breast cancer in mice. Am. J. Cancer, 36: 44–50 (1939).
(22) ———: Breast cancer in breeding and virgin "A" and "B" stock female mice and their hybrids. Pub. Health Rep., 54: 1113–1118 (1939).
(23) ———: "Influences" of breast-cancer development in mice. Pub. Health Rep., 54: 1590–1597 (1939).
(24) ———: Breast cancer and pedigree relationship of fostered A stock mice. Pub. Health Rep., 54: 1642–1650 (1939).
(25) BITTNER, J. J., and LITTLE, C. C.: Transmission of breast and lung cancer in mice. J. Hered., 28: 117–121 (1937).
(26) BITTNER, J. J., and MURRAY, W. S.: Comparative study of four high tumor lines of mice. Am. Nat., 70: 443–453 (1936).
(27) CASTLE, W. E.: Genetics and eugenics. Ed. 4. Harvard Univ. Press, Cambridge (1930).
(28) CLOUDMAN, A. M.: Gross and microscopic diagnoses in mouse tumors at site of mammary glands. Am. J. Cancer, 27: 510–512 (1936).
(29) CLOUDMAN, A. M., BITTNER, J. J., and LITTLE, C. C.: Relationship between histology of spontaneous mouse tumors and genetic constitution of animals in which they arise. Occas. Pubs. Am. A. Advancement Sc., 4: 37–41 (1937).
(30) CLOUDMAN, A. M., and LITTLE, C. C.: Genetics of tumour formation in mice in relation to gene T for brachyury. J. Genetics, 32: 487–504 (1936).
(31) DAVENPORT, C. B.: Heredity in relation to eugenics. Henry Holt & Co., New York (1911).
(32) DOBROVOLSKAIA-ZAVADSKAIA, N.: Heredity of cancer susceptibility in mice. J. Genetics, 27: 181–198 (1933).
(33) FEKETE, E.: Observations on three functional tests in a high-tumor and a low-tumor strain of mice. Am. J. Cancer, 38: 234–238 (1940).
(34) GREENE, H. S. N.: Heterologous transplantation of human and other mammalian tumors. Science, 88: 357 (1938).
(35) ———: Familial mammary tumors in rabbit; clinical history. J. Exper. Med., 70: 147–158 (1939).
(36) ———: Familial mammary tumors in rabbit; gross and microscopic pathology. J. Exper. Med., 70: 159–166 (1939).
(37) GREENE, H. S. N.: Familial mammary tumors in rabbit; factors concerned in their genesis and development. J. Exper. Med., 70: 167–184 (1939).
(38) ———: Familial mammary tumors in rabbit; evolution of autonomy in course of tumor development as indicated by transplantation experiments. J. Exper. Med., 71: 305–324 (1940).
(39) HAALAND, M.: Spontaneous tumours in mice. 4th Scient. Rep. (Gt. Brit.) Imp. Cancer Research Fund, pp. 1–113 (1911).
(40) HOLMES, S. J.: Human genetics and its social import. McGraw-Hill, New York (1936).
(41) ROSCOE B. JACKSON MEMORIAL LABORATORY STAFF: The existence of nonchromosomal influence in the incidence of mammary tumors in mice. Science, 78: 465–466 (1933).
(42) ———: The constitutional factors in the incidence of mammary tumors. Am. J. Cancer, 27: 551–555 (1936).
(43) KORTEWEG, R.: Chromosomale invloeden op den groei en extra-chromosomale invloeden op het ontstaan van kanker bij de muis. Nederl. tijdschr. v. geneesk., 79: 1482–1490 (1935).
(44) LATHROP, A. E. C., and LOEB, L.: Further investigations on the origin of tumors in mice. I. Tumor incidence and tumor age in various strains of mice. J. Exper. Med., 22: 646–673 (1915).
(45) LATHROP, A. E. C., and LOEB, L.: Further investigations on the origin of tumors in mice. II. Tumor incidence and tumor age in hybrids. J. Exper. Med., 22: 713–731 (1915).
(46) LITTLE, C. C.: The inheritance of a predisposition to cancer in man. *In* Eugenics, Genetics and the Family. Sci. Papers 2d Internat. Cong. Eugenics, New York, pp. 186–190 (1923).
(47) ———: Evidence that cancer is not a simple Mendelian recessive. J. Cancer Research, 12: 30–46 (1928).
(48) ———: The role of heredity in determining the incidence and growth of cancer. Am. J. Cancer, 15: 2780–2789 (1931).
(49) ———: Relation of coat color to spontaneous incidence of mammary tumors in mice. J. Exper. Med., 59: 229–250 (1934).
(50) ———: Hybridization and tumor formation in mice. Proc. Nat. Acad. Sc., 25: 452–455 (1939).
(51) LITTLE, C. C., MURRAY, W. S., and CLOUDMAN, A. M.: Genetics of non-epithelial tumor formation in mice. Am. J. Cancer, 26: 431–450 (1939).
(52) LYNCH, C. J.: Studies on relation between tumor susceptibility and heredity. J. Exper. Med., 39: 481–495 (1924).
(53) ———: Relation between tumor susceptibility and heredity; incidence of tar tumors in strains of mice having differing incidence of spontaneous growths. J. Exper. Med., 42: 829–840 (1925).

(54) LYNCH, C. J.: Studies on relation between tumor susceptibility and heredity; spontaneous tumors of lung in mice. J. Exper. Med., 43: 339–365 (1926).

(55) ———: Studies on relation between tumor susceptibility and heredity; influence of heredity upon incidence of lung tumors in mice. J. Exper. Med., 54: 747–760 (1931).

(56) ———: Studies on relation between tumor susceptibility and heredity; lung tumors in mice with respect to phenomenon of maternal influence. Am. J. Cancer, 31: 77–84 (1937).

(57) MACDOWELL, E. C.: What and why is leukemia?—A medico-biological study on mice. *In* Carnegie Institution of Washington, Cooperation in Research, Publ. No. 501, pp. 353–367 (1938).

(58) MCFARLAND, J., and MEADE, T. S.: Genetic origin of tumors supported by their simultaneous and symmetrical occurrence in homologous twins. Am. J. M. Sc., 184: 66–80 (1932).

(59) MACKLIN, M. T.: Hereditary factors in human neoplasms. Quart. Rev. Biol., 7: 255–281.

(60) ———: Sex incidence of entodermal tumors. Am. J. Surg., 21: 438–445 (1933).

(61) ———: Value of accurate statistics in the study of cancer. Canad. Pub. Health J., 25: 369–373 (1934).

(62) ———: Familial incidence of cancer. Univ. Wisconsin, Symposium on Cancer, pp. 32–45 (1938).

(63) MACKLIN, M. T., and MACKLIN, C. C.: Adenocarcinoma of large intestine in presumably dizygotic twin brothers. Arch. Path., 27: 133–137 (1939).

(64) MURRAY, J. A.: Cancerous ancestry and the incidence of cancer in mice. 4th Scient. Rep., (Gt. Brit.) Imp. Cancer Research Fund, pp. 114–131 (1911).

(65) MURRAY, W. S.: Breeding behavior of dilute brown stock of mice (Little dba). Am. J. Cancer, 20: 573–593 (1934).

(66) MURRAY, W. S., and LITTLE, C. C.: The genetics of mammary tumor in mice. Genetics, 20: 466–496 (1935).

(67) MURRAY, W. S., and LITTLE, C. C.: Further data on existence of extrachromosomal influence on incidence of mammary tumors in mice. Science, 82: 228–230 (1935).

(68) MURRAY, W. S., and LITTLE, C. C.: Extrachromosomal influence in relation to the incidence of mammary and nonmammary tumors in mice. Am. J. Cancer, 27: 516–518 (1936).

(69) MURRAY, W. S., and LITTLE, C. C.: Chromosomal and extrachromosomal influence in relation to the incidence of mammary tumors in mice. Am. J. Cancer, 37: 536–552 (1939).

(70) SLYE, M.: The incidence and inheritability of spontaneous cancer in mice. (Preliminary report). Ztschr. f. Krebsforsch., 13: 500–504 (1913).

(71) ———: Nature and heredity of cancer. J. Cancer Research, 10: 15–49 (1926).

(72) STRONG, L. C.: The establishment of the C_3H inbred strain of mice for the study of spontaneous carcinoma of the mammary gland. Genetics, 20: 586–591 (1935).

(73) ———: Genetic appearance of spontaneous carcinoma of mammary gland in C_3H mice. Am. J. Cancer, 25: 599–606 (1935).

(74) ———: Age distribution of 1250 spontaneous carcinomata of mammary glands in female mice of the A strain. Am. J. Cancer, 30: 527–529 (1937).

(75) TYZZER, E. E.: A study of heredity in relation to the development of tumors in mice. J. M. Research, 17: 199–211.

(76) ———: A study of heredity in relation to the development of tumors in mice. Caroline Brewer Croft Fund Cancer Commission, Harvard M. School Rep., 4: 71–83 (1907).

(77) ———: A series of spontaneous tumors in mice, with observations on the influence of heredity on the frequency of their occurrence. J. M. Research, 21: 479–518 (1909).

(78) WARTHIN, A. S.: Heredity with reference to carcinoma as shown by the study of the cases examined in the pathological laboratory of the University of Michigan, 1895–1913. Arch. Int. Med., 12: 546–555 (1913).

(79) ———: Further study of a cancer family. J. Cancer Research, 9: 279–286 (1935).

(80) WASSINK, W. F.: Cancer et hérédité. Genetica, 17: 103–144 (1935).

(81) WRIGHT, S.: On the genetics of subnormal development of the head (otocephaly) in the guinea pig. Genetics, 19: 471–505 (1934).

(82) ———: An analysis of variability in number of digits in an inbred strain of guinea pigs. Genetics, 19: 506–536 (1934).

Spontaneous Tumors in a Subline of Strain C3H Mice

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This paper reports the incidence of spontaneous tumors in a subline of strain C3H mice maintained at the National Cancer Institute. The strain was originated in 1920 by Strong (*1*) who mated a male of the Little dilute brown strain to a female albino mouse obtained from Bagg. Mice having the coat color of wild mice were selected as breeders. Through the kindness of Dr. Strong a few of these mice were procured in October 1930. They and their progeny were mated brother to sister until January 1933, at which time the colony came under its present supervision.

Details of the manner in which the line was obtained and the results of inbreeding up to February 1937 were presented in an earlier paper (*2*). During January 1933, 31 females were mated to their brothers; all were descendants from female No. 49230 and male No. 52478 of Strong's colony. After 5 generations of inbreeding, 2 lines were selected for further inbreeding; and after 5 more generations, 1 line was selected as the more susceptible to the development of spontaneous mammary-gland tumors. This line was chosen for propagation and has undergone over 30 generations of uninterrupted brother-to-sister matings. Only mice of this line are included in this report.

EXPERIMENTAL PROCEDURE

The primary purpose of the investigation was to develop a strain of mice highly susceptible to spontaneous mammary-gland tumors. After the selection of the line, further selection was made by discontinuing the breeding of any family in which successive generations with tumors were interrupted by the death of a mouse free from tumor or by the occurrence of a mammary tumor in a mouse over 10.5 months of age.

No change has been made in the breeding or handling of the mice since January 1933. They are fed Purina dog-chow pellets[1] as a standard diet; food and water are available at all times. Litters are weaned when 1 month of age, when all males except one are removed. This procedure leads to pregnancy at an early age. Pregnant females are removed from the breeding cage, and each is placed in a separate lying-in cage. From the time a female is placed in a lying-in cage up to the time her litter is weaned, she receives a supplementary diet of bread soaked in milk.

After a mouse has given birth to one or more litters she is kept under observation for the remainder of her life. Every breeding female mouse, regardless of whether her litter died at birth, or during lactation, or was successfully nursed and weaned, is included in table 1 which presents the mammary-tumor incidence in these mice.

Virgin females are separated from their male litter mates when weaned and have no opportunity to breed. The incidence of mammary tumors among these mice is presented in table 2.

[1] *According to the manufacturer the chemical analysis of the food is: Protein, 23 percent; fat, 5 percent; fiber, 4 percent. The mineral analysis is: Iron, 0.018 percent; magnesium, 0.09 percent; silica, 0.23 percent; potassium, 0.56 percent; sodium, 0.67 percent; chlorine, 0.68 percent; phosphorus, 1.17 percent; and calcium, 1.5 percent. The manufacturer also states that the food contains adequate amounts of vitamins A, B_1, and D, and of riboflavin.*

TABLE 1.—*Time of occurrence of spontaneous mammary-gland tumors or deaths in breeding females of the C3H strain of mice*

[Numerators = number of mice developing tumors]
[Denominators = number of mice dying free from tumor]

Generation	Number of mice	Number developing tumors or dying free from tumors according to age (in months)																					Tumor incidence	Average tumor age
		3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	Total	
																							Percent	*Months*
F	1														1/0								1/0	100	16.00
F_1	4									1/0			1/0		1/0				1/0				4/0	100	15.25
F_2	5				1/0			1/0			1/0			1/0					1/0				5/0	100	12.40
F_3	4							1/0			1/0	1/0			1/0								4/0	100	12.50
F_4	8		0/1					3/0	1/0	1/0	1/0		1/0										7/1	87.50	10.57
F_5	6			0/1			2/0	2/0						1/0									5/1	83.33	9.80
F_6	9	0/1		0/1		1/0	1/0	1/0	2/0	1/0	1/0												7/2	77.77	9.57
F_7	32		0/3	1/1	2/0	5/0	3/0	5/3	2/0	1/1	3/0	1/0								0/1			23/9	71.87	8.73
F_8	37	0/1	0/2	0/1	0/3	0/4	3/0	2/2	5/2	3/0	4/2	1/0	1/0	1/0									20/17	54.05	10.75
F_9	50			0/1	3/1	2/4	12/2	5/1	5/1	8/1				1/0			1/0		1/0			1/0	39/11	78.00	9.92
F_{10}	70			1/3	2/2	7/1	4/1	15/0	11/0	4/1	4/0	2/0	3/0	1/0	4/0	1/0			0/1	2/0			61/9	87.14	10.51
F_{11}	79			0/2	1/0	6/2	23/1	14/0	10/0	6/0	4/0	2/0	1/0	2/0	1/0	0/1							73/6	92.40	9.26
F_{12}	85					11/0	17/0	10/0	15/0	16/0	3/1	3/0	3/0	1/0	2/0	2/1							83/2	97.65	9.96
F_{13}	115			1/0	7/0	22/2	21/1	28/1	20/3	4/0		1/0			1/0								108/7	93.91	8.53
F_{14}	65			2/0	5/1	8/1	19/1	16/0	3/0	7/0	2/0												62/3	95.38	8.43
F_{15}	75			2/0	7/0	13/0	22/1	12/0	8/0	4/1	2/0	2/0					1/0						73/2	97.33	8.48
F_{16}	92	0/2		0/1	5/0	22/1	27/0	15/0	11/1	5/0	2/0												87/5	94.56	8.32
F_{17}	71			1/0	9/0	9/0	26/0	10/0	6/0	8/0			1/0						1/0				71/0	100	8.48
F_{18}	94			2/0	10/0	15/0	25/0	15/0	13/0	7/0	5/0	2/0											91/0	100	8.54
F_{19}	82		3/0	5/0	9/0	12/0	23/0	13/0	6/0	7/0	3/0		1/0										82/0	100	8.08
F_{20}	96		1/0	3/0	12/0	12/0	18/1	21/0	11/0	8/0	3/0	2/0	3/0	1/0									95/1	98.96	8.68
F_{21}	70	1/0		1/1	6/0	14/0	23/0	9/0	7/0	4/0	2/0	2/0											69/1	98.57	8.27
F_{22}	52			2/0	10/0	20/0	8/0	4/1	3/0	1/0	3/0												51/1	98.08	7.59
F_{23}	68			5/0	5/0	21/0	17/0	12/0	2/0	4/0		0/1	0/1										66/2	97.06	7.73
F_{24}	84		1/1	7/3	10/0	23/1	14/0	15/0	7/0	1/0	1/0												79/5	94.05	7.59
F_{25}	84			1/1	14/2	12/8	18/0	16/0	4/1	4/1	1/1												70/14	83.33	7.96
F_{26}	58	0/1	1/1	1/5	7/10	12/11	2/0	6/1															29/29	50.00	7.07
F_{27}	92	0/1	2/1	8/3	11/2	25/2	16/0	14/0	3/0	4/0													83/9	90.22	7.43
Total	1,588	1/6	8/9	43/24	139/21	272/37	347/8	265/9	155/8	109/5	46/4	19/1	15/1	9/0	11/0	3/2	2/0		4/1	2/1		1/0	1,451/137	91.37	8.58

TABLE 2.—*Time of occurrence of spontaneous mammary-gland tumors or deaths in virgin females of the C3H strain of mice*

[Numerators=number of mice developing tumors]
[Denominators=number of mice dying free from tumor]

Generation	Number of mice	Number developing tumors or dying free from tumor according to age (in months)																Tumor incidence	Average tumor age
		5	6	7	8	9	10	11	12	13	14	15	16	17	18	22	Total		
																		Percent	*Months*
F_{12}	12			1/0	1/0	2/0		2/0	3/0		1/0	1/0		1/0			12/0	100	11.42
F_{13}	6					1/0			1/0		2/0	2/0					6/0	100	13.17
F_{14}	59		0/1	5/0	5/0	10/0	9/0	7/0	8/0	5/1	1/0	2/0	3/0	2/0			57/2	96.61	10.86
F_{15}	57			1/0	7/0	11/0	6/0	8/0	7/1	4/0	2/1	4/2	1/0		1/1		52/5	91.23	10.92
F_{16}	12				2/0	3/0	1/0	3/0	1/0	1/0	1/0						12/0	100	10.42
F_{19}	22		2/0	3/0	5/0	4/0	4/0	2/0	2/0								22/0	100	8.86
F_{20}	33			2/0	4/0	5/0	5/0	7/0	5/0	5/0							33/0	100	10.39
F_{21}	66		4/0	1/0	7/0	12/0	11/0	7/0	11/0	9/0	1/0		1/0	2/0			66/0	100	10.53
F_{22}	23	1/0	1/0	2/0	2/0	5/0	3/0	6/0	1/0	1/0		1/0					23/0	100	9.65
F_{23}	14			3/0		5/0	2/0	2/0	1/0		1/0						14/0	100	9.57
F_{25}	46	2/0	4/0	2/0	2/0	5/0	10/0	12/1	1/0	1/0	4/0			1/0		0/1	44/2	95.65	9.98
Total	350	3/0	11/1	20/0	35/0	63/0	51/0	56/1	41/1	26/1	13/1	10/2	5/0	6/0	1/1	0/1	341/9	97.43	10.43

Both males and females were used to ascertain the occurrence of spontaneous hepatic and pulmonary tumors. The males were set aside for this purpose, and the presence of tumors of the liver and lung was determined when they died or when they were killed at various ages. Most of the females were virgins which had developed mammary tumors, but the majority of those 15 months of age or older had been foster nursed by mice of other strains. It is known that foster nursing does not influence the incidence of hepatomas [2] or of pulmonary tumors (*3*) in mice. The occurrence of spontaneous tumors of the liver and lung is summarized in table 3. The incidence of mammary, hepatic, and pulmonary tumors is also presented in graphic form in figure 1.

[2] *Andervont, H. B.: Unpublished data.*

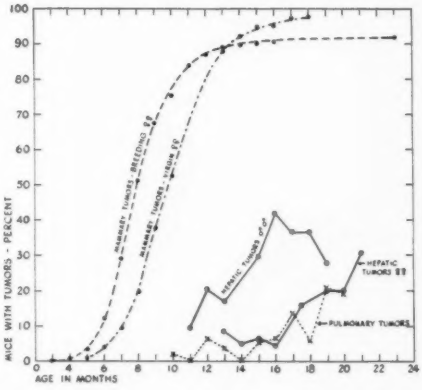

FIGURE 1.—Incidence of spontaneous tumors in a colony of strain C3H mice. Incidences of mammary tumors are cumulative (tables 1 and 2); incidences of internal tumors are charted as percentages of total mice killed at given age periods (table 3).

TABLE 3.—*Occurrence of spontaneous hepatic and pulmonary tumors in strain C3H mice*

Mice	Age (in months)																	Total
	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	
Female:																		
Number examined	12	21	28	40	13	23	20	15	24	11	6	5	5	13	2	2	2	242
Number with hepatic tumor	0	0	0	0	0	2	1	1	1	3	0	1	1	4	0	0	0	14
Number with pulmonary tumor	0	0	0	0	1	0	0	1	0	1	0	1	1	0	0	1	0	6
Male:																		
Number examined	5	2	22	21	34	53	12	146	24	11	11	29	----	----	----	----	----	370
Number with hepatic tumor	0	0	0	2	7	9	1	43	10	4	4	8	----	----	----	----	----	88
Number with pulmonary tumor	0	0	1	0	2	3	0	8	3	2	1	6	----	----	----	----	----	26

RESULTS

Mammary-Gland Tumors in Breeding Females

The results of 27 generations of inbreeding are presented in table 1. All generations up to the F_{27} generation are completed, that is, all the mice have developed tumors or have died from other causes. Ten mice of the F_{27} generation ranging from 8 to 10 months of age are alive.

It is apparent that this line of strain C3H mice exhibits a high incidence of mammary tumors. This is clearly shown in generations F_{17} to F_{23} inclusive, in which a very small number died without developing a tumor. The results in these generations indicate that practically 100 percent of breeding C3H females will develop spontaneous mammary tumors if they live until 14 months of age.

The conditions under which the mice were maintained exerted a definite influence upon the tumor incidence. From January 1933 to October 1939, they were kept in Boston, from January 1933 to June 1936 in a separate room at the Harvard Medical School, from June 1936 to June 1938 at the Bussey Institute, and from June 1938 to October 1939 at the Gibbs Memorial Laboratory. While at the Bussey Institute they had the highest incidence of tumors in successive generations (F_{17} to F_{23}), and the records reveal that during those 2 years only 19 died before developing tumor. During that time they were maintained under almost ideal conditions. They were not exposed to mice from outside sources and were handled by an attendant who never came into contact with other mice. The high mortality in the F_{27} generation occurred during October 1939 and accompanied the move from Boston to Bethesda, Md., where they are housed at the present time.

The last column of table 1 reveals that the average mammary-tumor age of breeding mice has shown a tendency to decrease in recent generations. This may be due to selection as described previously, to an increase in the number of litters born to each female, or to a combination of these influences. Prior to the F_{25} generation, most of the mice bore but one litter, and only in the recent generations have they raised more than one litter. Data from colony records are inadequate to demonstrate whether more intensive breeding alone lowers the average tumor age. An experiment to test this possibility is now in progress.

The 1,588 breeding females included in table 1 do not represent all breeding C3H females observed. At least an equal number of others have been used for experimental purposes and are not included in the table. These mice showed the same high incidence and low average-tumor age as those recorded here. The majority of strain C3H breeders develop multiple mammary tumors before death, but accurate data are not available because most of them were used for experimental purposes as soon as the first tumor appeared.

Mammary-Gland Tumors in Virgin Females

Table 2 summarizes the occurrence of spontaneous mammary tumors in virgin strain C3H females. In an earlier publication (*4*) data on 117 virgins were presented, and these are included in table 2. The virgin C3H females show the same high incidence of mammary tumors as do the breeding mice, but the tumors appear at a later average age. The incidence of 97.43 percent in 350 virgins is higher than the incidence of 91.37 in 1,588 breeders, but this does not mean the virgins are more susceptible to mammary cancer. The chance of survival among virgins is better

than among breeders, and this increased viability leads to more of the virgins living to the tumor age.

The figures in the last column of table 2 suggest that, as in the case of breeding females, the average tumor age of virgin C3H mice is decreasing. This may be regarded as evidence that selection is responsible for the decrease, but further observations are essential before a definite conclusion can be made.

Hepatic and Pulmonary Tumors

The incidence of spontaneous hepatic and pulmonary tumors in 284 C3H mice was recorded in a previous paper (*5*), and these are included in table 3. Recent efforts were directed towards ascertaining the occurrence of hepatomas in male mice over 1 year of age and especially in 15-months-old mice.

Table 3 shows that of 157 females from 11 to 19 months of age 9, or 5.7 percent, had hepatic tumors, while of 341 males of the same age group 88, or 25.8 percent, had similar growths. It is clear that male C3H mice exhibit a higher incidence of spontaneous hepatomas than do the females or that the tumors arise earlier in the males. Table 3 also shows that of the 141 females more than 1 year old, 9.93 percent developed spontaneous hepatomas whereas 4.25 percent developed pulmonary tumors; of the 320 males in this age group, 26.87 percent developed spontaneous hepatomas and 7.81 percent developed spontaneous pulmonary tumors.

Of the mice listed in table 3 only 6 females and 26 males had primary pulmonary tumors. This is not interpreted as showing that the males are more susceptible than the females. All pulmonary tumors in the females recorded in table 3 arose in mice which were free from mammary tumor since it was desirable to preclude that the growths were metastatic. Strain C3H mice apparently are less susceptible to pulmonary tumors than to hepatic tumors.

DISCUSSION

Strong reported on the incidence of spontaneous mammary tumors in strain C3H breeding females. In one paper (*1*) he recorded that of 42 females, 38, or 90.5 percent, developed tumors at an average age of 10 months, and in another (*6*) that of 83 females, 58, or 69.9 percent, developed tumors at an average age of 13.7 months. Bittner (*7*) found that of 200 breeding C3H females, 156, or 78 percent, developed mammary tumors, and Bittner and Murray (*8*) reported the average tumor age of these mice to be 11.4 months. Suntzeff, Burns, Moskop, and Loeb (*9*) observed an incidence of 60.8 percent at an average age of 10.9 months in 46 breeding C3H females, and Burns and Schenken (*10*) noted an incidence of 69.6 percent at an average age of 12.16 months in 46 breeding females. Burns and Schenken reported an incidence of 54.16 percent hepatomas in males and an incidence of 7.69 percent in females which were the offspring of strain C3H mice obtained from the Roscoe B. Jackson Memorial Laboratory.

Data presented here indicate that the C3H mice raised in this laboratory exhibit a higher incidence of mammary tumors and an earlier average age of appearance of the tumors than those described by the above-mentioned investigators. The mice of this line have been inbred for 10 years and have apparently developed their own substrain characteristics. Tumors arising in them do not grow progressively upon transplantation into C3H mice obtained from the Roscoe B. Jackson Memorial Laboratory. The colony is regarded as a separate line of C3H mice, and results obtained with it cannot be controlled by

data procured from other lines of strain C3H mice.

When compared with other well-known high-mammary-tumor strains of mice such as strain A (*11*) or strain dba (*12*), strain C3H mice are considered the most susceptible for the following reasons: (1) The breeding females exhibit practically a 100-percent incidence of mammary tumors at an average of age 8 to 9 months, while strain A and strain dba females do so at an average age of 10 to 11 months; (2) the mammary-cancer incidence is also practically 100 percent in virgin C3H females, whereas in strain A it is 5 percent, and in strain dba it is 50 percent.

Strain C3H females are useful in studies on the occurrence of spontaneous mammary cancer in mice. The high incidence supplies satisfactory controls for experiments on the inhibition (*13*) of the tumors, and the low average-tumor age is useful in ascertaining the influence of hybridization (*14*, *15*) upon the time of appearance of this type of tumor.

They are excellent experimental animals for investigating the influence of mother's milk upon the occurrence of mammary tumors in mice (*4*, *16*, *17*). Their genetic constitution is such that they are either extremely susceptible to a cancer-inciting material in mouse milk, or they produce or transmit considerable quantities of the material.

The relatively low average-tumor age is especially useful in foster-nursing studies because it shortens the time of the experiment. The results are usually evident within 7 months when breeding mice are employed and within 9 months when virgins serve as test animals. The breeding mice bear but one litter which is killed within 24 hours after birth. Further breeding is not necessary.

The strain also supplies animals for experiments on the therapy of mammary tumors. The chief objection here is their tendency to develop multiple tumors, but the tumors arise at an early age when the animals are vigorous and are able to withstand treatment better than older animals.

Female C3H mice, because of their high susceptibility to mammary tumors and consequent short life, are not satisfactory for experimental work which extends over long periods of time. The males, however, are long-lived and are used extensively; they are very susceptible to the induction of subcutaneous tumors when injected with carcinogenic hydrocarbons (*18*). The males are also susceptible to the induction of mammary tumors following injections of estrogens (*19*).

Both males and females develop spontaneous hepatomas. In mice over 1 year of age the males show an incidence of 26.87 percent and the females an incidence of 9.92 percent. Compared with other strains of inbred mice in which the incidence of hepatomas has been recorded (*5*, *10*), the C3H strain is a susceptible strain. Both males and females also develop spontaneous pulmonary tumors late in life, but they are less susceptible to pulmonary than to hepatic tumors. As compared with strain A mice (*11*), the C3H animals are fairly resistant to primary pulmonary tumors. Subcutaneous or intravenous injections of 1, 2, 5, 6-dibenzanthracene increase the incidence and hasten the time of appearance of primary pulmonary tumors (*5*) in C3H mice, but subcutaneous injections of 2-amino-5-azotoluene (*20*) or 3, 4, 5, 6-dibenzcarbazole [3] do not increase the incidence of hepatomas.

Other types of spontaneous neoplasia are uncommon, but old foster-nursed females develop cystic changes in ovaries and uteri.

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[3] *Andervont, H. B.: Unpublished data.*

Little trouble is encountered in maintaining the strain. They breed well, and the mothers take good care of their young. Practically all are fertile, and the females usually deliver their first litters when from 2 to 3 months of age. The average size of first litters is six while the second or third litters average eight. Few females raise more than three litters because they develop mammary tumors at an early age.

The chief difficulty is the sporadic occurrence of a disease which kills entire litters when they are from 10 to 14 days old. The young mice develop diarrhea and usually die within 24 hours. First litters appear to be more susceptible than later litters. The etiology of the disease is obscure, and efforts to control it have been unsuccessful.

SUMMARY AND CONCLUSIONS

The incidence of spontaneous tumors of the mammary gland, liver, and lung in a subline of strain C3H mice maintained at the National Cancer Institute is reported.

Of 1,588 breeding females, 91.37 percent developed spontaneous mammary-gland tumors at an average age of 8.58 months. Of 350 virgin females, 97.43 percent developed spontaneous mammary-gland tumors at an average age of 10.43 months.

Of 141 females over 1 year of age, 9.93 percent developed spontaneous hepatomas and 4.25 percent developed pulmonary tumors. Of 320 males over 1 year of age, 26.87 percent developed spontaneous hepatomas and 7.81 percent developed spontaneous pulmonary tumors.

Compared with other strains of inbred mice, this subline of strain C3H mice is highly susceptible to spontaneous mammary-gland tumors and spontaneous hepatomas but relatively resistant to spontaneous pulmonary tumors. Other types of spontaneous neoplasia are uncommon.

The colony is regarded as a separate line of C3H mice, and results obtained with it are not directly comparable with those procured with other lines of strain C3H mice.

REFERENCES

(*1*) STRONG, L. C.: The establishment of the C3H inbred strain of mice for the study of spontaneous carcinoma of the mammary gland. Genetics, 20: 586–591 (1935).

(*2*) ANDERVONT, H. B., and MCELENEY, W. J.: Incidence of spontaneous tumors in a colony of strain C3H mice. Pub. Health Rep., 52: 772–780 (1937).

(*3*) ANDERVONT, H. B.: Pulmonary tumors in mice. II. The influence of heredity upon lung tumors induced by the subcutaneous injection of a lard-dibenzanthracene solution. Pub. Health Rep., 52: 304–315 (1937).

(*4*) ANDERVONT, H. B., and MCELENEY, W. J.: The influence of nonbreeding and foster nursing upon the occurrence of spontaneous breast tumors in strain C3H mice. Pub. Health Rep., 53: 777–783 (1938).

(*5*) ANDERVONT, H. B.: The occurrence of spontaneous and induced pulmonary and liver tumors in strain C3H mice. Pub. Health Rep., 54: 1158–1169 (1939).

(*6*) STRONG, L. C.: The genetic appearance of spontaneous carcinoma of the mammary gland in the C3H mice. Am. J. Cancer, 25: 599–606 (1935).

(*7*) BITTNER, J. J.: The breeding behavior and tumor incidence of a black agouti stock of mice. Am. J. Cancer, 25: 614–620 (1935).

(*8*) BITTNER, J. J., and MURRAY, W. S.: Comparative study of four high tumor lines of mice. Am. Nat., 70: 443–453 (1936).

(*9*) SUNTZEFF, V., BURNS, E. L., MOSKOP, M., and LOEB, L.: The effect of injections of estrin on the incidence of mammary cancer in various strains of mice. Am. J. Cancer, 27: 229–245 (1936).

(*10*) BURNS, E. L., and SCHENKEN, J. R.: Spontaneous primary hepatomas in mice of strain C3H: A study of incidence, sex distribution and morbid anatomy. Am. J. Cancer, 39: 25–35 (1940).

(*11*) BITTNER, J. J.: Breast and lung carcinoma in "A" stock mice. Pub. Health Rep., 54: 380–392 (1939).

(*12*) MURRAY, W. S.: Sex hormones and cancer. Some effects of the interplay of sex hormones upon the incidence of mammary cancer in mice. Am. J. Cancer, 30: 517–526 (1937).

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(*13*) NATHANSON, I. T., and ANDERVONT, H. B.: Effect of testosterone propionate on development and growth of mammary carcinoma in female mice. Proc. Soc. Exper. Biol. & Med., 40: 421–422 (1939).

(*14*) ANDERVONT, H. B.: The incidence of induced subcutaneous and pulmonary tumors and spontaneous mammary tumors in hybrid mice. Pub. Health Rep., 53: 1665–1671 (1938).

(*15*) ———: Further studies on the susceptibility of hybrid mice to induced and spontaneous tumors. J. Nat. Cancer Inst., 1: 135–145 (1940).

(*16*) ANDERVONT, H. B., and MCELENEY, W. J.: The influence of foster nursing upon the incidence of spontaneous breast cancer in strain C3H mice. Pub. Health Rep., 54: 1597–1603 (1939).

(*17*) ANDERVONT, H. B.: The influence of foster nursing upon the incidence of spontaneous mammary cancer in resistant and susceptible mice. J. Nat. Cancer Inst., 1: 147–153 (1940).

(*18*) ———: Susceptibility of mice to spontaneous, induced and transplantable tumors. A comparative study of eight strains. Pub. Health Rep., 53: 1647–1665 (1938).

(*19*) SHIMKIN, M. B., and GRADY, H. G.: Carcinogenic potency of stilbestrol and estrone in C3H mice. J. Nat. Cancer Inst., 1: 119–128 (1940).

(*20*) ANDERVONT, H. B.: The susceptibility of five inbred strains of mice to liver changes induced by subcutaneous injections of 2-amino-5-azotoluene. Pub. Health Rep., 54: 1986–1991 (1939).

Changes in Cellular, Nuclear, and Nucleolar Sizes During Methylcholanthrene Epidermal Carcinogenesis[1]

By E. V. Cowdry, *professor of cytology, Washington University,* and *director of research, Barnard Free Skin and Cancer Hospital, St. Louis,* and F. X. Paletta, *research fellow,* in the same institutions

In an earlier paper we described changes in intranuclear viscosity in epidermal methylcholanthrene carcinogenesis in mice (Cowdry and Paletta (*1*)). The viscosity was found to be relatively high in normal epidermal cells, slightly lower in cells rendered hyperplastic by the treatment, and very much lower in active cells of the resulting squamous-cell carcinomas. In corresponding lesions spontaneously occurring in humans, similar alterations in intranuclear viscosity were observed. Our measure of viscosity was the extent of displacement of nuclear contents seen in the different tissues compared after subjection to equal ultracentrifugal force.

Since alterations in nuclear and cytoplasmic volume by methylcholanthrene treatment were quite noticeable, we decided to measure them as accurately as possible in like stages of carcinogenesis but, of course, in other mice. We thought that this would bring to light a relation, or a lack of relation, between the changes in viscosity and volume. Increase in volume might involve increase in water content which in turn might reduce viscosity.

Another reason why the work reported in this paper was undertaken is that the Barnard Hospital is attempting to determine the alterations that occur in as many properties as can be measured quantitatively in standardized methylcholanthrene carcinogenesis and to integrate the results with a view to the building of a comprehensive picture of the modifications that lead to cancer in epidermal cells.

Thus, investigations are being made by microincineration of certain mineral constituents of protoplasm. It would not be sufficient to determine simply the quantity, and to some extent the quality, of the nuclear or cytoplasmic ash at different stages. Obviously, such determination must be considered in relation to the volumetric changes that take place. If, for instance, the quantity of ash per nucleus were to remain the same during a certain period, the significance of this observation would depend to some extent upon whether during the same period the nuclei remained the same size or increased in volume. For the same reason no one would undertake permeability studies and ignore alterations in cellular volumes. It is difficult to mention any property which could be advantageously studied through the sequence of events without relation to the changing volumes of the cells.

Literature on cell size is voluminous. A hundred years ago Lehert made many measurements of cancerous and normal cells. He became convinced that the size of the cell and its nucleus afforded a valuable criterion of distinction between malignant and normal cells. His collected studies were published in book form in 1851. By this time, however, Virchow had checked some of Lehert's work and had announced in 1847 a different con-

[1] *Aided by a grant from the National Cancer Institute.*

clusion—that the size of cancer cells is not distinctive and that it is, therefore, of no value in diagnosis.

Today there is still marked difference of opinion on the results reported from the measurement of malignant and normal cells. Thus Ehrich (*2*) in summarizing his work and that of his students was very emphatic in the statement that "malignant tumors are characterized by doubling or quadrupling of the sizes of their nuclei"; while Shairer (*3*) could find no evidence of such clear-cut difference between malignant and normal tissues. MacCarty and his associates at the Mayo Clinic have investigated the nucleoli and have been publishing papers on the subject since 1925. He has reached the conclusion (*4*) that:

> The nucleolus is much larger in proportion to the size of the nucleus in all malignant cells, regardless of the type or origin of the neoplasm, this difference being great enough to permit easy differentiation from all other cells in the hands of one experienced with fresh tissues.

To name all MacCarty's supporters and those who have disagreed with him would consume too much space, but reference may be made to the favorable report of Von Haam and Alexander (*5*) and the dissenting opinion of Guttman and Halpern (*6*). It is difficult to avoid the feeling that this assertion of MacCarty is too sweeping and comprehensive. It almost invites diagnosis of malignancy on the basis of a single criterion.

A review in the American Journal of Cancer (vol. *16*, p. 1021, 1932) of Heiberg's paper on another measurement, that of the diameter of the equatorial plate in mitosis, is of interest. The reviewer says:

> Like the generations of cancer workers who have occupied themselves with the question (of cellular measurements), Heiberg is empty handed. Indeed it may be said that there has been no more futile field of cancer research than this one.

The chief difficulty has been lack of directness of the comparison between the malignant and normal cells (Cowdry (*7*)). To utilize cells rendered malignant experimentally and to compare them not only with their normal prototypes but also with intermediate stages, as Page (*8*) has done, is a step in advance. We shall have occasion to refer to his work frequently in the following account.

MATERIAL

Like Page, we employed a 0.3-percent solution of methylcholanthrene in benzene and we also applied it to the skin 3 times a week. He added 8–10 drops of the carcinogen to the backs of mice and hastened evaporation of the benzene with a blower, whereas we applied it in cotton on the end of a wooden applicator to the centers of the inner surfaces of the ears of 100 white mice of mixed stock. The carcinogen in our experiment spread over the entire inner surfaces of the ears before the benzene evaporated. Precautions were taken to avoid evaporation of the benzene in the stock solution, for this would obviously lead to progressive concentration of methylcholanthrene and interfere seriously with uniformity of treatment.

In the following list are given the days after the beginning of treatment when the tissues were removed and, in brackets, the number of mice from which the tissues were taken: 1 [2], 2 [2], 3 [2], 4 [2], 5 [2], 6 [2], 7 [2], 8 [2], 9 [2], 11 [2], 13 [2], 16 [2], 18 [1], 19 [5], 23 [1], 25 [2], 27 [4], 29 [1], 31 [1], 32 [2], 34 [2], 35 [1], 37 [1], 38 [1], 39 [2], 40 [1], 44 [1], 46 [2], 48 [1], 52 [2], 55 [1], 59 [1], 60 [1], 62 [2], 65 [2], 67 [1], 72 [2]. All were fixed in Bouin's fluid. Paraffin sections, cut 5μ in thickness, were stained with haematoxylin and eosin. Great care was taken to dehydrate,

clear, and imbed the tissues uniformly so that the influence of the technique on shrinkage would be fairly uniform.

The earliest microscopic evidence of malignancy was seen in specimens taken on the forty-sixth, sixty-second, and seventy-second days following the beginning of treatment. Our records show that 45 percent of the mice living at the end of 80 days developed squamous-cell carcinomas of the ears by the end of 120 days. This does not represent the actual number that would have developed malignant tumors because the ultimate fate, had they not been interfered with, of many nonmalignant lesions biopsied after 80 days cannot be predicted. Response to the carcinogen was as rapid and as regular as in the pure strains of mice afterwards employed in other experiments. After a close study of the whole series, we selected eight specimens as representative, namely, 0, 13, 19, 31, 39, 46, 62, and 72 days of treatment. On these our measurements were made.

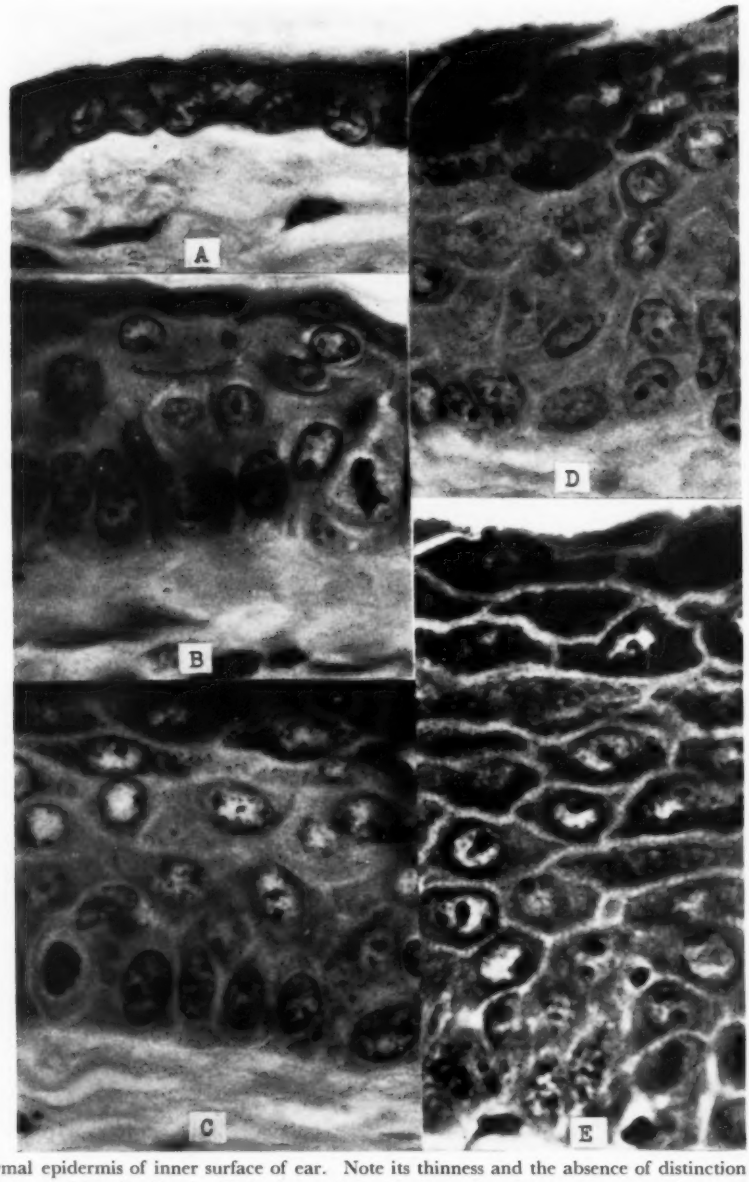
Photomicrographs (pls. 1 and 2) were taken of typical parts of each of the specimens. The sections passed through the centers of the ears vertical to their treated internal surfaces. Since, with increase in thickness of the epidermis, there is an increase in the number of nuclei per unit area, the measurement of the later and thicker specimens involved examination of a decreasing surface area as compared with the earlier and thinner ones. Hair follicles were not included in the measurements, neither were occasional patches of epidermis infiltrated by leukocytes and parts evidently traumatized. In the stages of hyperplasia the 800 nuclei measured were made up of 400 from basal cells and 400 from spinous cells. These two were kept separate because it is not positively known which are more directly in the line of production of squamous-cell carcinomas in mice. Separate counts of the incidence of mitosis were also made for the same reason. By "basal cells" are understood those in actual contact with the basement membrane.

How often the epidermal-cell population of the ear changes when treated with methylcholanthrene is obscure. While the population samples examined were obviously very small and were excised at rather infrequent intervals, many other preparations were surveyed to make sure that the samples chosen were truly representative of large numbers of cells.

The procedure in the measurements was to outline the nuclei on paper with the aid of a camera lucida at a magnification of 1,530 diameters. Because cell membranes were often indistinct, no attempt was made to outline them. Instead, the total background of the nucleated area was considered cytoplasmic. The error in this assumption was least in the case of normal epidermis, intermediate in the hyperplastic epidermis, and greatest in the carcinoma in proportion to the differences in volume of the spaces between the normal, hyperplastic, and malignant cells. Finally the outlines were transferred from the paper to No. 3 Kodaloid (Eastman Kodak Co.) by using a stylus pencil. The nuclear areas were cut out with curved scissors, and their combined weights were determined, as well as the weights of the Kodaloid representing cytoplasm, at each stage of the comparison.

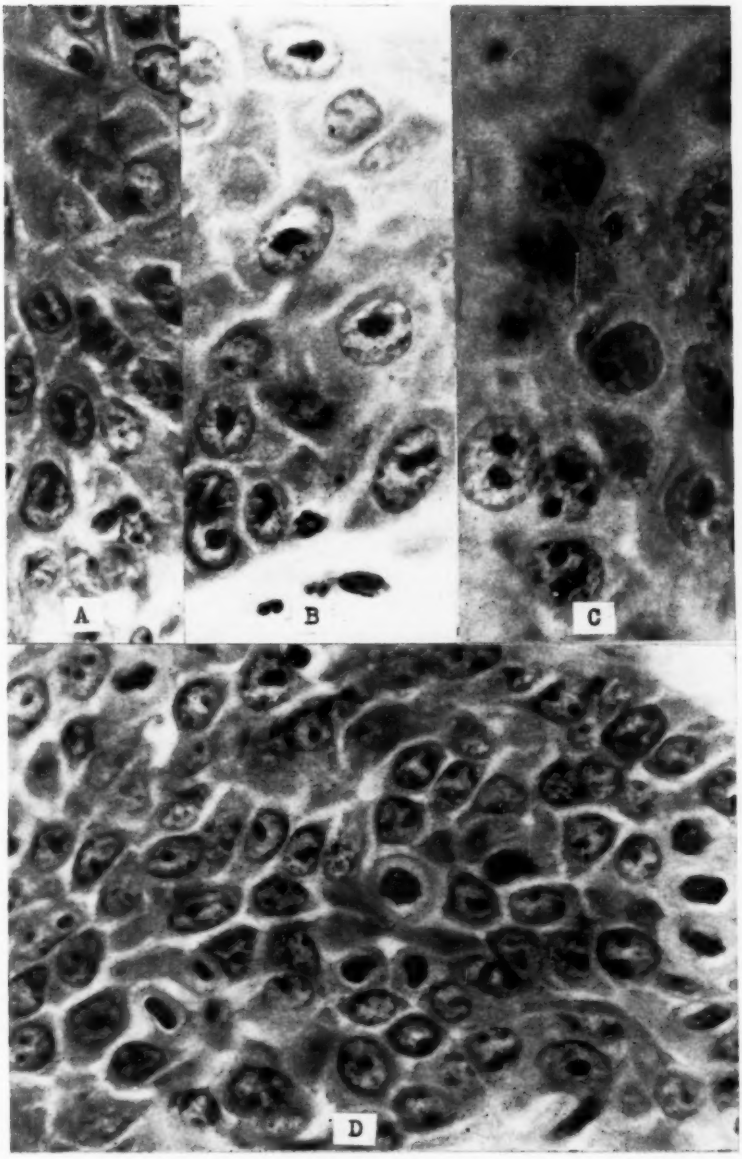
The nuclear computations were based on the assumption that the nuclear form approaches that of a prolate spheroid. The average nuclear volume was determined by a formula derived by Drs. G. H. Scott and V. Rojansky, of Washington University (personal communication). The formula is given as $1.5 \times \frac{S^2}{2A}$, where S is defined as the average area in cross section and A as the semi-major axis of the prolate

PLATE 1



A, Normal epidermis of inner surface of ear. Note its thinness and the absence of distinction between spinous and basal cells; *B*, Slight hyperplasia 13 days after first treatment with methylcholanthrene. Spinous cells can easily be distinguished from basal cells; *C*, Hyperplasia after 19 days. Marked increase in keratohyalin; *D*, Hyperplasia after 31 days; *E*, Hyperplasia after 39 days. Comparison with *A-D* shows increase in thickness of epidermis, in size of cells and nuclei in prominence of the processes of the spinous cells, and in amount of keratohyalin. All × 1040.

PLATE 2



A, Part of hyperplastic epidermis after 46 days. Marked increase in size and number of nucleoli. Measurement of cells in spinous layer revealed increase in nuclear size and slight decrease in cell size as compared with 39-day specimen (pl. 1, *D*); *B* and *C*, Portions of hyperplastic epidermis bordering a malignant area in a 62-day specimen. Marked increase in cytoplasm, *B*, and especially in the nucleoli, *C*. Determinations of nuclear and cytoplasmic volume largest obtained in this carcinogenic series. Nucleoli very large and multiple. *D*, Active area of squamous-cell carcinoma in a 72-day specimen. Mitosis frequent. Cells and nuclei are fairly uniform in respect to size relations. Sizes of cells, nuclei, and nucleoli distinctly smaller than in *B* and *C*, and nucleocytoplasmic ratio is much higher. All × 1040.

spheroid. The value, S, can be determined, if the weight of the Kodaloid representing the nuclei, the weight of 1 sq. cm. of the Kodaloid, the magnification, and the number of nuclei are known. The value $2A$ is obtained by measuring the long axis of the nuclei and computing the average major axis.

Estimates of nucleocytoplasmic and cellular ratios were made on the supposition that the cells contained only one nucleus. Search for binucleated cells showed them to be extremely rare. Although in another experiment a few small foci of multinucleated epidermal cells were observed, none were encountered in the particular series reported in this paper.

OBSERVATIONS

The early stages, from the beginning of treatment up to and including the twelfth day, were not investigated, but six carefully selected stages in the subsequent epidermal hyperplasia were studied in detail. A squamous-cell carcinoma, 72 days after the first application of methylcholanthrene, was chosen for detailed comparison. Our presentation, therefore, covers this period of hyperplasia and includes a comparison of malignant and hyperplastic cells, but it does not include an analysis of volumetric changes during the transition between hyperplastic and malignant cells.

Hyperplasia

Examination of plate 1 and plate 2, *A*, *B*, and *C*, shows that, in the areas of tissue presented from these specimens, there is a gradual increase in nuclear size up to the 62-day specimen (pl. 2, *B* and *C*). But the alterations in nuclear volume are better presented in figure 1. In this and in the other two graphs the measurements are plotted as ordinates and the periods of treatment in days as abscissas. Beginning on the left with the normal untreated epidermis, are given data on stages of hyperplasia ending with the 62-day specimen which is marked by a circle to indicate discontinuity with what follows. The last measurement, on the right, is of a squamous-cell carcinoma at 72 days. The lines between data from the last specimen of hyperplasia (62 days) and from the carcinoma are incomplete near the former because the stages of transition from hyperplastic to malignant cells are unknown. The upper broken line represents measurements of spinous cells, and the lower solid one those of basal cells. The two lines start at a common point representing nuclear volume in the untreated epidermis, because these two types of cells could not be distinguished in the normal epidermis of the ears of mice. They converge to a common point, representing the cancer at 72 days, because we did not wish to attempt a similar distinction among the malignant cells.

The sequence of changes suggested by figure 1 does not indicate that the nuclear enlargement measured in the 13-day specimen necessarily increases to that found in the 19-day specimen and so on throughout the series. In reality each

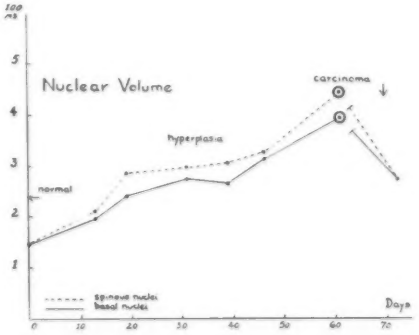


FIGURE 1.—Changes in volume of spinous and basal nuclei of cells of epidermis in transition from normal to malignant state.

and every connecting line should be incomplete on this and on the other two figures. It is by no means certain, for instance, that the degree of response observed in a 13-day specimen would become altered to conform with that determined in a 46-day specimen if the 13-day specimen had not been excised but had been allowed to progress 33 days longer before examination. In other words, the rate of response is not sufficiently uniform in different individuals to render a sequence based on days of treatment more than a probable one in which exceptions may be brought to light by further work. Later experiments convince us that variations in speed of response to the carcinogen cannot be entirely eliminated even in closely inbred strains of mice. Although nuclear volumes were not ascertained in other specimens, the sizes of nuclei in them were estimated by visual impression, and it is our belief that the nuclear volume gradually increases with duration of treatment and degree of resulting hyperplasia.

Nucleoli were not actually measured; but, since plate 1, *E*, at 39 days, plate 2, *A*, at 42 days, and plate 2, *B* and *C*, at 62 days show much larger nucleoli than those earlier in the series, it is possible that the increase in nucleolar size is progressive and that it parallels to some extent at least the increase which we have described in nuclear volume.

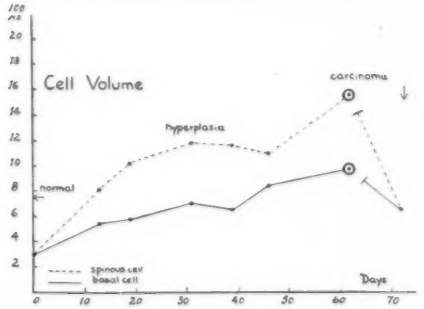


FIGURE 2.—Changes in volume in spinous and basal cells in transition from normal to neoplastic state.

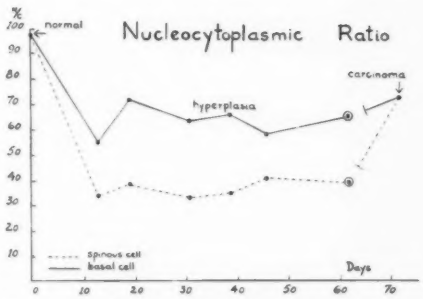


FIGURE 3.—Nucleocytoplasmic ratio of spinous to basal cells at various stages of transition from normal to neoplastic.

Instead of making direct measurements of cell volumes these were determined from data collected on nuclear and cytoplasmic volumes. They show (fig. 2) that, in the specimens examined, the increase in cell volume was progressive and somewhat similar to that of nuclear volume, except that the 39-day specimen showed a slight decrease, not only in the basal cells (below) but also in the spinous cells (above), as compared with those in the hyperplastic epidermis of 31 days. There was, moreover, a slight decrease in spinous cells of the 46-day specimen. Again, as is to be anticipated from the properties of these two periods in cellular differentiation, the volumetric increase was greater for the spinous cells than for the basal cells. The reduction in cell volume with the malignant change was in this particular case proportionally much larger than the reduction in nuclear volume.

By employing the data on nuclear and cytoplasmic volumes in spinous and basal cells, it was a simple matter to calculate nucleocytoplasmic ratios. For purposes of plotting curves, a single figure was obtained by dividing the nuclear volume by the cytoplasmic volume and multiplying by 100. The results are given in figure 3.

In the intermediate stages of hyperplasia the nucleocytoplasmic ratio for both spinous and basal cells decreased from the normal sharply, then fluctuated, and finally rose. The level during hyperplasia was higher for the basal cells, as can be seen from plate 1, *A–E*, and plate 2, *A–C*.

Because all the figures given for volumes are averages of many determinations, it is possible that a sharply localized change in a few cells might be masked by including with them many others which do not change in the same way. It was not feasible to calculate the variability in cytoplasmic volume because the cytoplasmic areas were not measured individually but were taken collectively as the area not occupied by nuclei. But all the nuclei studied were carefully outlined. Working with half the sums of the greatest diameter and the next greatest diameter, at right angles to the first, of each of the 400 basal and 400 spinous nuclei in every specimen (except the first and the last in which this distinction of cell type was not made), the mean values and the standard deviations were calculated and are presented in table 1.

TABLE 1.—*Nuclear variability in hyperplastic epidermis*

Tissue	Period of treatment	Mean value ± standard error of mean		Standard deviation	
		Basal cells	Spinous cells	Basal cells	Spinous cells
Normal epidermis	*Days*	6.17±0.088		1.76	
	13	6.85±0.080	6.86±0.072	1.60	1.44
	19	6.92± .089	7.03± .080	1.76	1.59
	31	7.24± .087	7.29± .084	1.74	1.68
	39	7.18± .090	7.38± .088	1.80	1.76
	46	7.67± .116	7.56± .098	2.30	1.96
	62	7.72± .126	7.76± .131	2.50	2.62
(Carcinoma)	72	6.74±0.084		1.68	

The standard deviations of the greatest diameters of basal and spinous nuclei in table 1 increased progressively during the stages of hyperplasia from 13 to 62 days, inclusive, which means that there was, during this period, in these specimens, a slight increase in range of variability in nuclear size. At 62 days the standard error of the mean was about 12 times that of normal epidermis. Visual examination of the same specimens also gave the impression of increasing variability.

A further analysis of this variability was made to ascertain its symmetry or lack of symmetry about the mean values. In table 2, the figures to the right indicate size values. For convenience these are not the actual nuclear sizes in microns. They are measurements in millimeters of the outlines of the nuclei magnified 1,530 times. Each number from 4 to 20 is half the sum of greatest nuclear diameter outlined and the next greatest diameter at right angles to the first. In the first and the last specimens no distinction was attempted between basal- and spinous-cell nuclei for reasons already given. In all, the number of nuclei having the mean value for size, thus measured arbitrarily in millimeters, is given in heavy type. The numbers of nuclei of each of the sizes less than the mean are inserted in the left of the mean, while the numbers of those of the sizes greater than the mean are to the right of the mean.

In the normal epidermis, addition of the number of nuclei to the left, and smaller than the mean, gives 121 as compared with the 206 larger nuclei to the right. This shows that the variability is not symmetrical about the mean but exhibits skewness, and is in the direction of larger size. The numerical distribution of sizes for all other nuclei, except for the hyperplastic spinous nuclei of the 19-day specimen, likewise indicates variability in the direction of larger size.

In other series extremely large, usually solitary, epidermal cells, with single nuclei five to eight times as large as those of their neighbors were seen in rare cases. Had

TABLE 2.—*Analysis of variability in nuclear size*

Length of treatment	Tissue	Numerical distribution of variation in nuclear size																
		4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
Days	Normal epidermis	1	1	19	32	68	**73**	106	54	28	14	3	1						
13	Basal			4	12	22	66	**89**	109	62	27	7	2						
	Spinous			5	12	45	**76**	107	81	48	21	4	1						
19	Basal		1	3	15	38	38	**79**	100	78	34	10	4						
	Spinous			3	10	25	41	80	**104**	94	34	7	2						
31	Basal			3	5	20	39	77	**100**	81	42	24	8	1					
	Spinous			2	5	18	34	73	**100**	88	46	27	6	1					
39	Basal			5	11	21	42	**65**	92	82	58	18	6						
	Spinous		1	3	6	20	32	**47**	95	100	63	26	6	0	1				
46	Basal			5	12	14	39	46	**74**	70	59	38	28	8	2	3	2		
	Spinous			1	10	16	40	44	**68**	86	72	38	20	4	0	0	1		
62	Basal	1	0	4	8	19	47	46	**64**	48	57	48	33	14	7	1	2	1	
	Spinous		1	1	6	19	54	51	**59**	53	55	43	24	14	7	8	1	3	1
72	Squamous-cell carcinoma	1	1	5	8	35	75	**90**	85	67	25	6	1	1					

they been present among the cells measured in this series the variation in nuclear size would have been very much greater. There is no reason to consider such isolated hypertrophied cells as an essential stage in the sequence.

In order to localize the site of most rapid cell division, in stages of epidermal hyperplasia, the incidence of mitosis was determined in 500 nuclei of basal cells, in an equal number of nuclei of spinous cells in the first layer distal to the basal cells, and in a squamous-cell carcinoma of 72 days.

The figures in table 3 indicate that the impetus to hyperplasia operates mainly in the basal layer. If we set aside the 46-day specimen, it is doubtful whether the incidence of mitosis was significantly different in the other five samples of hyperplastic epidermis.

TABLE 3.—*Incidence of mitosis*

Period of treatment	Tissue	Layer	
		Basal	Spinous
Days			
0	Normal epidermis	0	0
13	Hyperplastic epidermis	5	0
19	do	5	2
31	do	2	1
39	do	7	2
46	do	15	5
62	do	5	1
72	Squamous-cell carcinoma	36	0

SQUAMOUS-CELL CARCINOMAS

Figure 1 shows, not an increase, but a decrease in nuclear volume, and plate 2, *D*, illustrates a decrease in nucleolar size in a squamous-cell carcinoma, as compared with plate 2, *A–C*, of hyperplastic epidermis. Because this only applies to the area studied of this particular carcinoma, it was clearly necessary to investigate other carcinomas and to make the comparison with hyperplastic epidermis as definite and clear-cut as possible.

From a large collection of methylcholanthrene squamous-cell carcinomas of mice, 17 specimens were selected. With each was included some hyperplastic epidermis in which the differentiation of the cells was orderly and signs of malignancy were absent. A study was made of basal cells resting on the basement membrane and spinous cells in a single row immediately distal to them. Care was taken to exclude from the comparison hyperplastic epidermis in which the cells showed marked diversity in size, shape, and staining reaction, and in which the usual cellular stratification was lacking or notably irregular. To be specific, "anaplastic cells" like those represented by Szodoray (*9*) in his figure 7 of keratosis senilis as malignant were not included.

In the category of malignant cells selected for this comparison were cells which had broken through the basement membrane of the epidermis or had developed from other cells which did so and had invaded the dermis where they occurred singly or in small groups. Not included were cells in broad projections which stretch down into the dermis and may even displace or erode muscle when a fairly sharp line of demarcation exists between such epidermal tissue and the dermal tissue invaded, even though the component cells may look anaplastic. Neither were cells included that are present in the centers of dense epithelial clumps and consequently removed somewhat from the blood supply. Such cells, though developed from invasive cells, are often in the process of keratinization. In other words, their degree of differentiation, owing, perhaps, as in normal epidermis, to deprivation of vital necessities, sets them apart from malignant cells here studied. All obviously degenerating cells were likewise excluded. In short, in an arbitrary way the category of malignant cells was narrowed down to those which had infiltrated the dermis and which were active in the sense that they were relatively unspecialized and showed no signs of degeneration. This was done without any intention of reaching a definition of malignancy. Its sole purpose was to focus in this comparison hyperplastic cells and fairly definite groups of malignant cells, which groups we have characterized sufficiently for other investigators to locate similar ones in their own preparations. Every carcinoma in our series shows such regional diversity in cellular properties, which tends to increase as it progresses, so that only confusion would result if one compared malignant cells in a general way with hyperplastic cells.

No attempt was made to measure slight differences in a necessarily small fraction of the cellular population, but rather to determine by close microscopic study of many cells whether average sizes of malignant and hyperplastic cells were larger, smaller, or about the same. In the 17 carcinomas examined, the malignant cells were larger in 6, but smaller in 11, as compared with the spinous cells; and larger in 12, and the same size in 5, when compared with the basal cells. For the most part, the nuclei of malignant cells were larger (larger in 10, smaller in 6, and same size as 1, when compared with spinous nuclei; and larger in 11, smaller in 3, same size as 3, when compared with basal nuclei). The nucleocytoplasmic ratio was noticeably larger in the malignant cells in all 17 specimens than in the spinous cells, and about the same size as that in the basal cells. The nucleoli of the malignant cells were larger and more numerous in 12, and smaller in 5, as compared with those of both spinous and basal cells. But in neither cell size, nuclear size, nucleolar size, nor nucleolar number did the malignant cells in all of the 17 specimens differ uniformly the same way from the nonmalignant cells.

A similar comparison was made of human tissues except that the 32 squamous-cell carcinomas chosen, with attached hyperplastic epidermis, were graded. Of the 32, 8 were placed in grade I, 9 in grade II, 10 in grade III, and 5 in grade IV (table 4)

Evidently in the human tissues, as in the mouse tissues, no single cellular measurement is so characteristic of malignant cells as to differentiate them invariably from the neighboring hyperplastic epidermal cells, with the possible exception of their larger nucleocytoplasmic ratio as compared with that of the spinous cells.

The observations indicate unmistakably that when, in the carcinogenic series, the

TABLE 4.—*Comparison of malignant aud hyperlastic cells of humans*

Comparison of—	Grade and number in grade			
	I (8) [1]	II (9) [1]	III (10) [1]	IV (5) [1]
Malignant cells with spinous cells.	Smaller in 8	Larger in 1 Smaller in 8	Smaller in 10	Smaller in 5.
Malignant cells with basal cells.	Larger in 1 Same in 7	Larger in 2 Same in 7	Larger in 7 Same in 3	Larger in 2. Smaller in 2. Same in 1.
Malignant nuclei with spinous nuclei.	Larger in 5 Smaller in 2. Same in 1	Larger in 5 Smaller in 4	Larger in 7 Smaller in 3	Larger in 1. Smaller in 4.
Malignant nuclei with basal nuclei.	Larger in 5 Same in 3	Larger in 7 Same in 2	Larger in 9 Same in 1	Larger in 4. Same in 1.
Malignant nucleocytoplasmic ratio with spinous.	Larger in 8	Larger in 9	Larger in 10	Larger in 5.
Malignant nucleocytoplasmic ratio with basal.	Same in 8	Same in 9	Same in 10	Same in 5.
Malignant nucleoli with spinous nucleoli.	Larger, more numerous in 4. Smaller in 4	Larger, more numerous in 9.	Larger, more numerous in 8. Smaller in 2	Larger, more numerous in 5.
Malignant nucleoli with basal nucleoli.	Larger in 3 Smaller in 1 Same in 4	Larger, more numerous in 9.	Larger, more numerous in 8. Smaller in 2	Larger, more numerous in 1. Smaller in 2. Same in 2.

[1] Number in grade in parentheses.

cells exhibit behavior known as malignant, the previous mechanism of regulation of cellular-size relations no longer operates to maintain the same uniformity. Each resulting carcinoma is almost a law unto itself. In some carcinomas, like the 72-day specimen (table 1 and pl. 2, *D*), the sizes of the cells, nuclei, and nucleoli are fairly uniform and smaller than those of hyperplastic epidermal cells. In others the sizes may be uniformly larger. In some, however, the range in sizes is very marked, and within a single carcinoma many differences may occur. Growth, regulated in a single pattern in the hyperplastic epidermis, gives way to growth more or less regulated in a variety of patterns in the carcinomas.

DISCUSSION

Our observations differ from those of Page in certain particulars. He concluded that the nuclei responded to methylcholanthrene—

> by an immediate enlargement to one and two-thirds normal size, which was maintained until malignancy occurred, when the nuclei were again increased in size.

We question the immediacy of the response because the first specimens examined by Page were taken 48 hours after the first application of the carcinogen and our first was even later. The measurements we made did not reveal simply the maintenance of an enlargement observed on the thirteenth day through the specimens examined up to and including the one on the sixty-second day, but a definite and apparently progressive increase. The malignant cells in some carcinomas contained larger nuclei than those of the hyperplastic spinous and basal cells, while in others they were smaller.

Reporting on the nucleoli, Page described—

> an immediate increase in size, which reached a maximum ot from two to three times normal in one week; then there was a decrease in size from the maximum, but the nucleoli remained about twice normal size until a malignant lesion occurred; next the nucleoli increased in size.

Our preparations showed merely an increase in nucleolar size in the later stages of hyperplasia and in some carcinomas larger nucleoli, and in others smaller nucleoli, than in hyperplastic cells.

The size of the epithelial cells was in his opinion "increased throughout the period

of application of the carcinogenic agent." Whether this means that the size was immediately increased beginning with the first application and was then maintained at a dead level to the end, or whether the increase continued to grow greater throughout the period is not clear. In the stages of hyperplasia which we examined, the increase was progressive until malignancy occurred, when some carcinomas exhibited larger malignant cells and others smaller cells than the cells of a 62-day specimen of hyperplastic epidermis.

That Page and the present writers based their work on the same kinds of cells is doubtful. He referred only to basal cells, whereas we considered both basal and spinous cells separately. In the carcinomas he limited his observations to "characteristic cells, each with a single nucleolus." It was not possible for us to select strictly comparable malignant cells because we did not know what he regarded as characteristic. To exclude cancer cells with more than a single nucleolus seemed to us to be an artificial distinction which would eliminate many malignant cells that actually have more nucleoli than are seen when only a section of each nucleus is examined.

In some respects our observations supplement the results of our previous work on changes in intranuclear viscosity during carcinogenesis. The decrease in intranuclear viscosity from the condition observed in normal epidermal cells to that in late stages of hyperplasia was found to be small and gradual in the specimens studied. The increase herein reported in nuclear volume, though larger in degree, was also gradually progressive, and there is reason to think that it was roughly related in time to the alteration in viscosity despite the fact that it was not feasible to determine viscosity and volume in identical cells. The much more marked decrease in intranuclear viscosity in the malignant cells as compared with hyperplastic ones as yet differentiating in an orderly way, was correlated, only in a majority of the carcinomas, with increase in nuclear volume, because in some carcinomas the nuclei were smaller. We have no data to present on alterations in water content of the nuclei, but it is possible that increase in volume is accompanied by increase in water content and that this may be one factor in the decrease in intranuclear viscosity. However, in the skin near a carcinoma we found evidences of edema in both the dermis and epidermis, and it was difficult to displace the nuclear contents by centrifugal force which indicated high viscosity; but there were no signs that the nuclei shared in the fluid increase.

In addition to a low intranuclear viscosity, the malignant cells usually exhibited a high nucleocytoplasmic ratio. In embryonic epidermal cells the intranuclear viscosity was also low, and the nucleocytoplasmic ratio, though we did not actually measure it, was evidently fairly high. In untreated epidermal cells on the other hand, the intranuclear viscosity was high and the nucleocytoplasmic ratio was likewise high. Evidently, therefore, a low intranuclear viscosity is not incompatible with a high nucleocytoplasmic ratio.

According to Voegtlin (*10*), the heart of the cancer problem is to ascertain how any single carcinogenic agent causes the transformation of normal into malignant cells. There is much difference of opinion as to the action on cell division of methylcholanthrene. Earle and Voegtlin (*11*) have described growth inhibition in tissue cultures of fibroblasts after a latent period of a little over 5 days with no sign of stimulation at any time. However, employing the same carcinogen, Reimann and Ham-

mett (*12*) reported an increase in the growth of *Obelia geniculata*. The literature on tar cancer, 1927 to 1931, is reviewed by Seelig and Cooper (*13*). Since all of the conflicting accounts cannot well be explained on the basis of differences in experimental conditions, concentration of the agent, and so forth, the possibility presents itself that a single carcinogen may conceivably act differently on different cells.

In our series we did not make counts of the incidence of mitosis in early stages; but in a 13-day hyperplasia and in all the specimens taken thereafter, the incidence of mitosis per 1,000 nuclei was greater than in the normal, untreated epidermis. Whether this increase in mitosis is owing to direct action by the methylcholanthrene or results from a sequence of events initiated by it, we do not know. It is important to discover whether the daily rhythm in mitotic frequency, found in normal mouse epidermis (Carleton (*14*), Cooper (*15*)), is modified during carcinogenesis.

The factors directly concerned in the hyperplasia have not been brought to light. One may not suppose that, in view of efforts to achieve uniformity, the repeated "kicks" delivered by the carcinogen are of equal force throughout the series. Unless there is some kind of summation, the effectiveness of each application will decrease. This decrease will occur *pari passu* with increase in protection of the cells in contact with, or near the basement membrane provided by the shielding afforded by increase in thickness and keratinization of the layers distal to them. We now realize that the tissues should always have been excised at exactly the same time after the last application of carcinogen. During the interval between applications, it is possible that conditions in the tissue may change. Tissues excised 1 day after the last application, for example, may perhaps differ from those excised a day later.

The increase in number of cells in thickening epidermis is occasioned by decrease in the normal rate of loss of cells as well as by increase in frequency of cell division. The fact that in hyperplastic epidermis the cells continue to live at a much greater distance from the blood supply than they normally do in the same region, suggests a better provision for income of nutriment and elimination of waste (Cowdry (*16*).) When we compared an epidermis made hyperplastic by methylcholanthrene with an epidermis normally possessing about the same number of cells per unit area (soles of feet of mice), we found many interesting differences between them, some suggestive of a larger water content in the former. A preliminary comparison with regenerative epidermal hyperplasia in preparations made in this laboratory has also brought to light significant differences. Whether changes in water content may relate to changes in mineral content is being studied by Dr. W. Simpson. Hyperplastic epidermis is in many respects a favorable tissue for investigation. Detailed comparison of methylcholanthrene hyperplasia, which is definitely precancerous, with hyperplastic cells that do not ordinarily undergo malignant change will give some idea of the environment in which some cells undergo the malignant transformation and may supply clues as to the nature of the alteration.

A major task is to trace the actual transitions that occur when, out of a very numerous population of hyperplastic cells, a few cells, or perhaps only a single cell, becomes malignant. If this were not extremely difficult, it would have been accomplished long ago. Until it is done, we shall remain in the dark concerning the most significant steps in the sequences of

events. It is necessary to include as possible sources of malignant cells not only the basal and spinous cells of the general epidermal surface but also the epithelial cells of hair follicles and conceivably in rare instances those of sebaceous glands. Search is profitable only in lesions in which the malignant cells are beginning to appear, because after the carcinoma can be diagnosed by gross inspection the transitions may have become obscured. That is, the malignant cells in a full-blown carcinoma may not look the same as those first formed. The latter may conceivably take origin not only from hyperplastic groups of cells but also from atrophic areas. It is possible that local atrophy may, in some cases, follow hyperplasia. The method that holds out the most promise of success is to apply the carcinogen to an extremely small area, to select likely lesions, and to reconstruct them from serial sections.

SUMMARY

In mouse epidermis examined before and 13, 19, 31, 39, 46, and 62 days after the first methylcholanthrene treatment, a progressive increase was observed in nuclear and cytoplasmic volumes of both basal and spinous cells, but in a carcinoma at 72 days there was a marked decrease in both nuclear and cytoplasmic volumes. In the same specimens the nucleocytoplasmic ratio was high in the untreated normal epidermis, decreased during hyperplasia, and increased again in the carcinoma. The variability in nuclear size increased slightly during hyperplasia and decreased in the carcinoma. Judged by inspection of specimens and not by actual measurement, an increase in nucleolar size and number took place in the later stages of hyperplasia and a decrease in both in the carcinoma. Mitotic counts showed a higher incidence of mitosis in hyperplastic basal cells than in hyperplastic spinous cells or in untreated epidermal cells, but it was less than in the malignant cells.

Seventeen other methylcholanthrene squamous-cell carcinomas of mice were selected for examination, because it was unsafe to base conclusions on a single one. The malignant cells in each were compared with hyperplastic epidermal cells included in the same sections. In the majority of the carcinomas, they were smaller than the hyperplastic spinous cells but slightly larger than the hyperplastic basal cells; likewise in a majority the nuclei were larger. The nucleocytoplasmic ratio was distinctly larger in all of the carcinomas than in the spinous cells and about the same as in the basal cells. In the malignant cells of most of the specimens the nucleoli were larger and more numerous than in either spinous or basal cells. But in neither cell size, nuclear size, nucleolar size, nor number did the malignant cells in all of the 17 carcinomas differ from the nonmalignant ones. To employ cell size, or the size of any of these components, as a single diagnostic criterion of malignancy in these tumors of mice is therefore unjustifiable.

A similar comparison was made in 32 human squamous-cell carcinomas. In a majority the malignant cells were smaller than the hyperplastic spinous cells but of equal or larger size than the hyperplastic basal cells. In a majority also their nuclei were larger than those of spinous and basal cells. The nucleocytoplasmic ratio was conspicuously larger in the malignant cells than in the hyperplastic spinous cells and about the same size as that in the hyperplastic basal cells. In most of the specimens the nucleoli were larger and more numerous than in the spinous and basal cells. In these human tissues no single cellular measurement made yielded results so characteristic of malignant cells as to differentiate them invariably from

neighboring hyperplastic epidermal cells, with the possible exception of their larger nucleocytoplasmic ratio as compared with that of spinous cells.

REFERENCES

(*1*) COWDRY, E. V., and PALETTA, F. X.: Alterations in nuclear viscosity during experimental carcinogenesis determined by ultracentrifugation. Am. J. Path., 17: 335–357 (1941).

(*2*) EHRICH, W. E.: Nuclear sizes in growth disturbances. With special reference to the tumor cell nucleus. Am. J. M. Sc., 192: 772–790 (1936).

(*3*) SCHAIRER, E.: Die Beziehungen von Korngrösse und Geschwulstwachstum, untersucht am experimentellen Teerkrebs. Ztschr. f. Krebsforsch., 45: 279–297 (1937).

(*4*) MACCARTY, W. C.: The value of the macronucleolus in the cancer problem. Am. J. Cancer, 26: 529–532 (1936).

(*5*) VON HAAM, E., and ALEXANDER, H. G.: Cytological studies of malignant tumors. Am. J. Clin. Path., 6: 394–414 (1936).

(*6*) GUTTMAN, P. H., and HALPERN, S.: Nuclear-nucleolar volume ratio in cancer. Am. J. Cancer, 25: 802–806 (1935).

(*7*) COWDRY, E. V.: Properties of cancer cells. Arch. Path., 30: 1245 (1940).

(*8*) PAGE, R. C.: Cytologic changes in the skin of mice during application of carcinogenic agents. Arch. Path., 26: 800–813 (1938).

(*9*) SZODORAY, I.: Histologic characteristics of the so-called precancerous processes of the skin. Arch. Dermat. & Syph., 36: 552–560 (1937).

(*10*) VOEGTLIN, C.: Some chemical aspects of the cancer problem. Science, 88: 41–48 (1938).

(*11*) EARLE, W. R., and VOEGTLIN, C.: The mode of action of methylcholanthrene on cultures of normal tissues. Am. J. Cancer, 34: 373–390 (1938).

(*12*) REIMANN, S. P., and HAMMETT, F. S.: Proliferation stimulating action of 1 : 2 : 5 : 6-dibenzanthracene on Obelia geniculata. Am. J. Cancer, 23: 343–349 (1935).

(*13*) SEELIG, M. G., and COOPER, Z. K.: A review of the recent literature of tar cancer (1927–1931 inclusive). Am. J. Cancer, 17: 589–667 (1933).

(*14*) CARLETON, A.: A rhythmical periodicity in the mitotic division of animal cells. J. Anat., 68: 251–263 (1934).

(*15*) COOPER, ZOLA: Mitotic rhythm in the epidermis of the mouse. Anat. Rec., 78: 1–8 (1940).

(*16*) COWDRY, E. V.: A textbook of histology... Ed. 2, Lea & Febiger, Philadelphia (1938).

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Length of Survival of Mice With Induced Subcutaneous Sarcomas

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In many investigations on the induction of tumors in mice by means of carcinogenic polycyclic aromatic hydrocarbons, the animals usually are killed relatively soon after tumors appear at the site of injection. This is necessary because autolysis makes tissues from animals which have been dead for a number of hours unsatisfactory for detailed histologic examination. It prevents, however, certain observations which are of interest and which may be of importance.

This report is concerned with preliminary determinations of the length of survival of mice after the appearance of palpable sarcomas at the site of injection of carcinogenic hydrocarbons, and the relation of the survival time to the type and the dose of the carcinogenic hydrocarbon employed.

EXPERIMENTAL PROCEDURE

Male mice of strains C3H and L were obtained from the Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine. They were maintained under similar environmental conditions, and on a diet of Purina dog-chow pellets, with an unlimited supply of water. The animals were approximately 3 months old when the experiments were begun.

The carcinogens employed were 20-methylcholanthrene[1] (m. p. 178.5°–179.2° C., corrected) and 1,2,5,6-dibenzanthracene[2] (m. p. 267.2°–267.4° C., corrected), each dissolved in tricaprylin (f. p. 8.3°–9.0° C., corrected). All animals received a single subcutaneous injection into the right axilla, as follows: 27 C3H males, 0.5 mg. of methylcholanthrene in 0.25 cc. of tricaprylin; 50 C3H males, 0.1 mg. of methylcholanthrene in 0.1 cc. of tricaprylin; 26 C3H males, 0.5 mg. of dibenzanthracene in 0.25 cc. of tricaprylin; 26 C3H males, 0.1 mg. of dibenzanthracene in 0.05 cc. of tricaprylin; and 83 strain L males, 0.2 mg. of methylcholanthrene in 0.1 cc. of tricaprylin.[3] The number of mice in each group, as given in the tables, is corrected for extraneous mortality during the course of the experiment.[4]

Each animal was examined weekly for the presence of a palpable hard mass at the site of injection. As soon as such a mass was felt, the mouse was marked individually and placed in a separate cage where it was kept until death.

RESULTS

The appearance of subcutaneous tumors, as evinced by palpation, and the death of mice with the tumors are recorded in table 1. The data are recapitulated graphically in figures 1, 2, and 3, which show the cumulative frequencies of tumors in the respective tumor populations at weekly intervals.

[1] *Referred to as methylcholanthrene hereafter.*

[2] *Referred to as dibenzanthracene hereafter.*

[3] *Variations in the volume of solvent occur because the groups do not represent a single experiment but are control groups of several experiments on the effect of colchicine on the induction of sarcomas in mice, which were negative and are not reported.*

[4] *The derivation of corrected totals is given in detail elsewhere (1).*

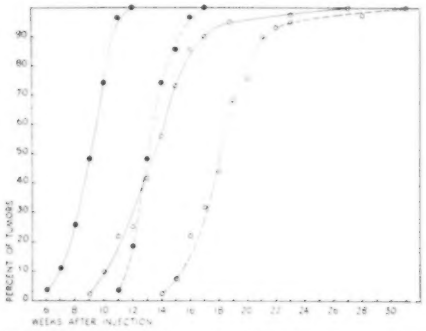

FIGURE 1.—Detection of palpable tumors and deaths of C3H male mice with tumors after subcutaneous injection of 0.5 or 0.1 mg. of methylcholanthrene in tricaprylin.

Solid line, solid circles = palpable tumors, 0.5 mg.

Broken line, solid circles = deaths, 0.5 mg.

Solid line, open circles = palpable tumors, 0.1 mg.

Broken line, open circles = deaths, 0.1 mg.

With the possible exception of the results with 0.1 mg. of dibenzanthracene (fig. 2), the curves for both the appearance of palpable tumors and deaths of animals are sigmoid in shape, and the curves for the deaths of the animals are parallel in each case with the curves for the appearance of palpable tumors. Table 1 indicates that the intervals between the means of these parallel curves are 4.3, 4.6, 4.2, 4.9, and 4.0 weeks.

The distribution of the survival times of the mice in each group is given in table 2. The mean survival times calculated by this regrouping are 4.15, 4.41, 4.02, 4.70, and 4.46 weeks, respectively. These differences are not statistically significant, i. e., the observed differences between the various

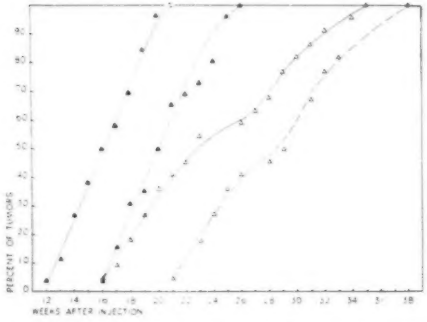

FIGURE 2.—Detection of palpable tumors and deaths of C3H male mice with tumors after subcutaneous injection of 0.5 or 0.1 mg. of dibenzanthracene in tricaprylin.

Solid line, solid triangles = palpable tumors, 0.5 mg.

Broken line, solid triangles = deaths, 0.5 mg.

Solid line, open triangles = palpable tumors, 0.1 mg.

Broken line, open triangles = deaths, 0.1 mg.

TABLE 1.—*Appearance of palpable tumors and death of mice following subcutaneous injection of methylcholanthrene or dibenzanthracene*

Strain of mouse	Hydrocarbon	Dose	Number of mice	Tumors or deaths of mice (in weeks after injection)	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	Test tumors	Average latent time
		Mg.																																				*No.*	*Weeks*
C3H	Methylcholanthrene	0.5	27	Tumors	1	2	4	6	7	6	1																											27	9.4±0.29
				Deaths						1	4	8	7	3	3	1																						13.7± .33
C3H	do	.1	46	Tumors				1	3	5	1	7	6	7	5	2	2				1				1													41	14.3± .51
				Deaths								1	2	6	4	5	10	3	6	1	1					1			1									18.9± .49
C3H	Dibenzanthracene	.5	26	Tumors							1	2	4	3	3	2	3	4	3	1																		26	16.6± .52
				Deaths											1	3	4	1	4	4	1	1	2	4	1														20.8± .59
C3H	do	.1	26	Tumors											1	1	2	2	2	1	1	2			1	1	1	2	1	1	1		1		1			22	24.4±1.23
				Deaths																1		3	2	2	1		1	1		4	2	1					4		29.3±1.12
L	Methylcholanthrene	.2	75	Tumors					1	1	4	2	2	4	6	5	8	4	4	4	2	2			1		1											51	17.5± .59
				Deaths								1		1	1	1	8	7	4	3	8	4	4	2	1		2	2			2								21.5± .56

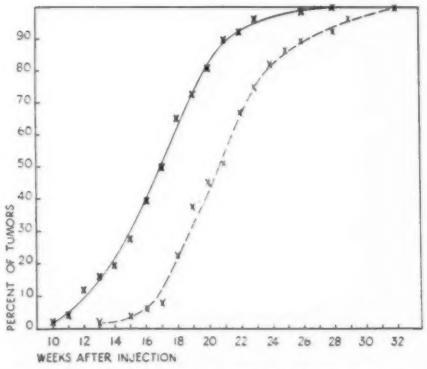

FIGURE 3.—Detection of palpable tumors and deaths of strain L males with tumors after subcutaneous injection of 0.2 mg. of methylcholanthrene in tricaprylin.
Solid line=palpable tumors.
Broken line=deaths.

means are in all cases less than two times the standard error of the differences. With both hydrocarbons, however, C3H mice that developed tumors after the injection of 0.1 mg. had a slightly longer mean survival time than the mice that received 0.5 mg. It is possible that a significant trend may be encountered with a wider separation in dosage.

In figure 4 the survival of individual animals was plotted against the time after injection of the hydrocarbon that the palpable tumor was detected. From the lack of a definite trend shown by the plotted points, and by the low value of the correlation coefficient ($r=-0.05$; $P>0.1$), it can be concluded that the length of survival after the appearance of a palpable tumor was not dependent upon the length of time from the original injection to the appearance of the palpable tumor.

The mean length of survival of mice after the appearance of palpable subcutaneous sarcomas, therefore, was the same whether methylcholanthrene or dibenzanthracene was used, whether the dose was 0.1 mg. or 0.5 mg., and whether the palpable tumors appeared early or late after the injection. The mean length of survival was the same in both strain C3H and strain L animals.

Since the differences in the length of survival were not significant, the 167 mice

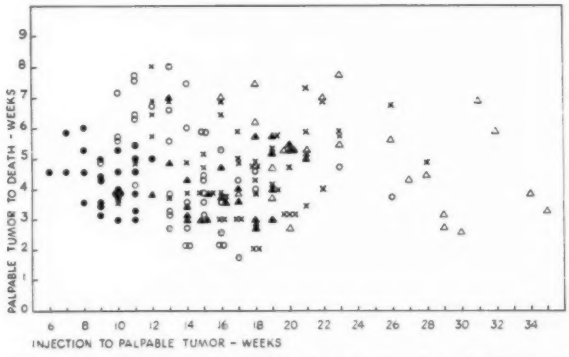

FIGURE 4.—Survival of individual animals after detection of palpable tumor according to weeks after injection of hydrocarbon that palpable tumor was detected.
Solid circles=0.5 mg. methylcholanthrene (27 C3H males).
Open circles=0.1 mg. methylcholanthrene (41 C3H males).
Solid triangles=0.5 mg. dibenzanthracene (26 C3H males).
Open triangles=0.1 mg. dibenzanthracene (22 C3H males).
Crosses=0.2 mg. methylcholanthrene (51 L males).

TABLE 2.—*Survival of mice following appearance of induced subcutaneous sarcomas, according to carcinogen and dose*

Strain of mouse	Carcinogen	Dose	Tumors	Survival according to time from palpable tumor until death (in weeks)														Mean survival
				1.5	2.0	2.5	3.0	2.5	4.0	4.5	5.0	5.5	6.0	6.5	7.0	7.5	8.0	
		Mg.	*Number*															*Weeks*
C3H	Methylcholanthrene	0.5	27				5	6	3	5	6	1	1					4.15±0.16
C3H	do	.1	41	1	4	3	3	6	5	3	1	5	3	2	2	2	1	4.41± .27
C3H	Dibenzanthracene	.5	26			1	8	4	3	2	5	2			1			4.02± .21
C3H	do	.1	22			3	2	3	2	1	3	2	1	1	3	1		4.70± .34
L	Methylcholanthrene	.2	51		2	1	8	8	4	9	4	6	2	5	1		1	4.46± .19
	Total		167	1	6	8	26	27	17	20	19	16	7	8	7	3	2	4.36± .10

were combined in order to obtain a better estimate of the mean for the whole series. This was found to be 4.36 ± 0.10 weeks.

It was of interest to compare the mean latent periods obtained in these groups of animals with the latent periods determined in a previous experiment performed under comparable conditions over a year ago (*2*). With 0.5 mg. of methylcholanthrene, the agreement was excellent—9.5 weeks in the previous series, and 9.4 weeks in the present group. With 0.5 mg. of dibenzanthracene, however, the previous mean latent period of 18 tumors was 23.3 ± 1.05 weeks, whereas in the present group it was 16.6 ± 0.52 weeks. The observed differences (6.7 weeks) was 5.7 times the standard error of the difference, or highly significant statistically. The reasons for this discrepancy are not apparent.

PATHOLOGIC OBSERVATIONS

Two mice, one injected with 0.1 mg. of methylcholanthrene and one with 0.1 mg. of dibenzanthracene, developed 2 discrete tumors at the site of injection. The remaining 165 mice developed single tumors.

In 2 mice not included in the results, the diagnosis of tumor by palpation was erroneous. In one the mass proved to be an oil cyst and in another a hemorrhagic lymph node. All of the 167 tumors which comprised the series grew progressively. Regression of the tumors was not observed, except that decrease in size occurred occasionally after ulceration and discharge of the necrotic contents of the mass.

The cause of death in the majority of the animals was attributable to the bulk of the tumor. The mass became so large that the mice moved and reached food and water with difficulty. Necrosis of varying degrees was present in practically all the tumors. Over one-fourth of the mice developed ulceration of the neoplasm, and in about 5 percent there was hemorrhage from the tumor.

Ninety-nine tumors were examined microscopically. All were classified as sarcomas. The tumors were locally invasive and often compressed the thorax and encompassed the ribs, but no penetration into the thoracic or abdominal cavities was observed. No distant metastases were noted, grossly.

DISCUSSION

The observations permit the conclusion that, within the limits of the experimental procedures, the length of survival of mice after the development of a palpable, induced subcutaneous sarcoma does not depend either upon the type of carcinogenic hydrocarbon or upon the dose of the agent employed. This is in agreement with the general opinion that the behavior of a tumor is independent of its inciting cause; i. e., once the tumor is established, the

carcinogen does not influence the course of the neoplasm.

The survival time is independent of the latent period between the injection of the hydrocarbon and the appearance of a palpable tumor. By the criterion of survival, then, tumors that appear early are no more "malignant" than those that appear at a protracted time after the introduction of the carcinogen.

The biologic behavior of induced subcutaneous sarcomas in mice is uniform in the ability to kill the host. In mice of strains C3H and L, maintained under the conditions of the experiment, the mean survival time after the appearance of a palpable tumor is 4.36 ± 0.10 weeks. The specific conditions of the experiment are emphasized, since it is known that many factors, particularly the diet (*3*, *4*, *5*), influence the growth of tumors and therefore necessarily the survival of animals with the tumors.

The parallelism between the palpatory detection of subcutaneous sarcomas and the death of the animals with these tumors indicates that the latter can be used as a criterion for the determination of the latent period of the tumors. In this manner, the carcinogenic potency of a compound can be measured by its delayed and indirect lethal effect.

SUMMARY

The length of time between the detection of induced subcutaneous sarcomas by palpation and death of the animal was studied in male mice of strains C3H and L.

The mean length of survival, 4.36 ± 0.10 weeks, was the same whether 20-methylcholanthrene or 1,2,5,6-dibenzanthracene was used, whether the dose of the carcinogen was 0.1 or 0.5 mg., and whether palpable tumors appeared early or late following the injection of the carcinogen.

Acknowledgement is made of the technical assistance of Mr. W. F. Gately.

REFERENCES

(*1*) Bryan, W. R., and Shimkin, M. B.: Quantitative analysis of dose-response data obtained with carcinogenic hydrocarbons. J. Nat. Cancer Inst., 1: 807–833 (1941).

(*2*) Shimkin, M. B., and Andervont, H. B.: Comparative carcinogenicity of three carcinogenic hydrocarbons. J. Nat. Cancer Inst., 1: 57–62 (1940).

(*3*) Voegtlin, C., and Thompson, J. W.: Lysine and malignant growth. I. The amino acid lysine as a factor controlling the growth rate of a typical neoplasm. Pub. Health Rep., 51: 1429–1436 (1936).

(*4*) Voegtlin, C., Johnson, J. M., and Thompson, J. W.: Glutathione and malignant growth. Pub. Health Rep., 51: 1689–1697 (1936).

(*5*) Bischoff, F., and Long, M. L.: The influence of low temperature environment on the growth of mouse sarcoma 180. Am. J. Cancer, 35: 86–89 (1939).

A Survey of Radiation Protection In Hospitals

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INTRODUCTION

There is an ever-increasing use of roentgen-ray equipment for medical purposes. New diagnostic procedures requiring the use of radiographic equipment have been devised, and the old procedures are being employed more frequently. Therapy techniques have been improved, and innumerable new uses for roentgen rays in the treatment of a wide variety of diseases have been found. While there has been a relatively greater increase in use of roentgen ray as compared with radium therapy, there has been an absolute increase in the use of radium for therapeutic purposes. The cyclotron has entered the picture in the last few years as a source of radiation for direct therapy and for production of radioactive compounds for treatment of disease.

This development of the cyclotron has also stimulated an increased interest in nuclear physics and has opened new fields to physicists and biologists. There are more than 16 cyclotron laboratories in the United States today, and through their development an increasing number of persons find themselves in situations where special precautions must be taken to prevent overexposure.

Industry has found the use of roentgen rays valuable as an aid in detecting defects in fabricated and other materials. Certainly the tremendous increase in production of military aircraft, naval vessels, and armament which is a part of our National Defense program will require even more extensive use of roentgen rays as time goes on.

The problems of protection of radiation workers must not be ignored as the use of radiation increases. The earliest workers did not know that biological reactions would occur as a result of exposure to roentgen rays, but they soon discovered some of its effects. Marcuse (*1*) reported a case of dermatitis and alopecia after illumination experiments with roentgen rays in 1896. The first cancer in man induced by exposure to roentgen rays was reported by Frieben in 1902, according to Colwell and Russ (*2*).

There has been a steady increase in recognition of the types of injury resulting from continued exposure of workers to roentgen rays and radium. Skin injury, including malignant tumor production, blood changes, sterility, cancer of the lung, osteoradionecrosis, and bone cancer are all well-recognized effects. Leukemia may be another one of the injuries, although it has not been considered as a result of overexposure as commonly as have the effects mentioned earlier. Nielson (*3*) found an excessive incidence of the disease in Denmark as shown by mortality records, and Furth and Furth (*4*) have described its eightfold greater incidence in a group of radiated mice than in a nonirradiated control series.

Genetic changes induced by exposure of *Drosophila*, mice, and plants to high-energy radiation have been described by many authors during the last 5 years, and this subject is reviewed in this Journal by Henshaw (*5*). The full implication of these findings in radiogenetics is not clear as yet in the case of man, but since the

genetic mechanisms of man and these other organisms are thought to be similar, they should cause enough alarm to necessitate their very careful consideration in planning protection until such time as it may be conclusively shown that they are unimportant.

Various amounts of radiation have been set as "tolerance doses" for roentgen-ray and radium workers by protection committees. These vary from 0.1 to 0.2 r per day to the entire body. The commonly accepted standard in the United States is 0.1 r, but in reality little is known of the amount and timing of radiation necessary to cause the commonly recognized injuries. Much attention has been focused on blood changes as an index of exposure, and it is partly on this basis that the present official tolerance doses were set, with full recognition that they are nothing more than arbitrary guides. While exposure of the entire body to less than 0.1 r daily may not cause blood changes, it may cause other local damage. Genetic effects may have no threshold, and it is believed the number of changes increases with increase in dose above zero.

The National Cancer Institute Act authorized and directed the Surgeon General of the United States Public Health Service to purchase radium and loan it to institutions for cancer research or for treatment of patients. Accordingly, 9½ gm. of radium were bought in 1938 and were loaned to 48 hospitals in various parts of the United States.

Because the work of the National Cancer Institute centers in medical problems, attention was concentrated primarily on protection as it is provided in the medical field. The great majority of radiation workers are employed in medical activities, however, and procedures and devices for protection which will shield them will also safeguard the nonmedical users of radiation.

SURVEY METHODS

A few hospitals were visited in the spring of 1940, and practices were observed in several of them which pointed to the need for making the survey on a more extensive basis. Since it was necessary that most of the institutions to which radium had been sent be visited with reference to the renewal of the loans for a second year, it was decided, for purposes of convenience, that the cooperation of this group would be requested in the conduct of the survey. Without exception, interest and willingness to cooperate were signified, and the authors wish to express appreciation, in behalf of the National Cancer Institute and themselves, to the many people who gave much of their time and permitted access to their equipment, thus making this survey possible.

After the preliminary visits mentioned, 45 other hospitals were visited, which are located in 24 States, scattered over the entire country. Twenty of them are State, county, city, or city-county institutions; 20 are operated by nonprofit associations, and the remaining 5 are church-owned. All are general hospitals except 3 which are a cancer hospital, a skin-and-cancer hospital, and a radiological institute, respectively. Twelve of the institutions are university hospitals, and 3 more are teaching hospitals affiliated with medical schools. They are relatively large, 32 of them having over 200 beds. Table 1 shows the distribution of beds in the institutions visited. These facts are pointed out in order to show that this is a group of hospitals in which one might expect to find typical, if not slightly above average, equipment and practices in matters of protection against high-energy radiation.

TABLE 1.—*Distribution of beds in the hospitals visited*

Beds	Hospitals	Beds	Hospitals
	Number		*Number*
Under 100	4	700–799	1
100–199	9	800–899	3
200–299	15	900–999	0
300–399	1	1,000–1,999	1
400–499	4	2,000–2,999	0
500–599	3	3,000–3,999	1
600–699	3		

Storage and handling of radium, calibration and shielding of roentgen-ray therapy equipment, and techniques of use and shielding of radiographic and roentgenoscopic units were the principal things studied in the hospitals visited. Two string electrometers and a group of ionization chambers of the Victoreen type, ranging in sensitivity from 0.25 to 100 r full scale, were employed in obtaining exposure and calibration data. By the use of the 0.25-r chambers, relatively accurate measurements of amounts of radiation as low as 0.01 r were made. Two electrometers were used so that one could serve as a check on the other. All instruments were calibrated at the start of the survey and were rechecked repeatedly during its course.

Estimates of average exposures of personnel were calculated from ionization measurements made during typical operations such as preparation of radium applicators and roentgenoscopic and radiographic procedures, multiplied by the average daily time spent by the individual performing such activities. Adequacy of equipment and practices were judged in most instances on the basis of suggestions for protection which have been published by various authors (*6–10*), and the unified set of safety recommendations agreed upon by the Advisory Committee on X-ray and Radium Protection, published in the National Bureau of Standards handbooks on X-ray (*11*) and radium (*12*) protection.

RADIUM

STORAGE

The amount of lead or lead equivalent necessary for suitable storage depends on (1) amount of radium to be stored, (2) the distance from the radium to the nearest person, and (3) the period of time that the person remains at this distance.

It is impossible to make recommendations on the exact storage-block thickness for varying amounts of radium that would be useful in all institutions unless one allows a generous safety factor for variations in place of storage and proximity of personnel.

A copy of the handbook on radium protection mentioned earlier and a list of supplementary recommendations were sent to the institutions to which Federal radium was shipped. On the supplementary list was a table of recommended lead or lead-equivalent thickness for storage containers for different amounts of radium under average conditions. These are approximately one-third more than the amounts necessary to allow a tolerance dose of 0.1 r in an 8-hour working day at a distance of 5 feet. The following tabulation shows these recommendations. Using it and the working habits of the people involved as a basis, the authors rated the storage methods of the various hospitals and found that 16 were excellent, 13 were intermediate, and 16 allowed definite overexposure.

Amount of radium	*Lead thickness*
100 mg	3.81 mm. (1½ in.)
200 mg	5.08 mm. (2 in.)
300 mg	5.71 mm. (2¼ in.)
400 mg	6.35 mm. (2½ in.)
500 mg	6.98 mm. (2¾ in.)

Eleven of the forty-five hospitals visited stored their radium in main-business-office safes. This place was used because of the intrinsic value of the radium, the fact that these safes offered the best safe-

guard against theft, and because the superintendent or an office clerk could be made responsible for checking the radium as it was taken out or returned, thus helping to prevent its loss. In a few of these cases the amout of lead was adequate, but in many it was not and varied from one-eighth to one-half inch in thickness, with amounts of radium stored in excess of 100 mg. and as high as 500 mg. In these same instances stenographers often worked from 7 to 8 hours within 5 to 6 feet of the radium.

The use of main-business-office safes created another exposure hazard in several cases. As a rule, the night supervisor of nurses did not have the combination of the safe; hence, when radium was removed from a patient after the close of business hours, it often was kept in the nurse's desk inadequately protected. In other cases the radium was locked in wooden or steel drawers in the office to await the arrival of the hospital superintendent or someone else who would place it in the safe. In one instance, because of the failure to take 125 mg. out of the drawer the first thing in the morning, a number of people worked near it for a half day without protection. In several additional hospitals the people responsible for the radium in the office admitted that they were often very busy and failed to put it away. In one of these hospitals, 150 mg. of radium in two small lead cylinders, each less than one-quarter inch in effective thickness and containing 75 mg., was returned at 9 in the morning by a nurse and was given to a stenographer who, because she was very busy and had not been instructed in the dangers of exposure to radium, placed the cylinders on her desk instead of putting them in the safe where there was an adequate lead container. She had been exposed for 5 hours on the occasion when the authors discovered the situation and stated that she often kept the radium on her desk but did not know that there was any danger of overexposure since it was in lead containers. In hospitals where adequate lead carriers were used, the storage of the radium in the office safe did not lead to overexposure, because the radium was placed in the carrier when it was removed from a patient at night.

Radium was stored in main operating rooms in which a number of people were on almost continuous duty during most of the day in two hospitals; however, in these two the lead containers were adequate for protection. In seven others, minor operating rooms were used as storage places, and protection was adequate in all but two of these. Radiologists' offices were used in three instances, and protection in these was adequate. In eight cases special rooms were used exclusively for storage and assembly of radium. Protection was adequate in all but one, and in this case 500 mg. of radium was stored and assembled beside an ordinary plaster-and-steel-lath wall on the opposite side of which was a desk at which a stenographer sat for 6 hours each day. She was 5 feet from the radium during this time and received a tolerance dose each hour she spent at the desk.

In the remaining 14 hospitals, storage was in a variety of places such as tumor clinics, roentgen-ray therapy rooms, patients' dressing rooms, and workrooms on operating room floors. In most of these the lead of the storage container was adequate, and the fact that there were no people working continually in close proximity to the radium gave an additional safeguard.

While the lead thickness of the storage container was adequate in many instances when the radium units were stored unassembled, the practice of placing assembled

applicators outside the lead safe because the space in the lead container was not large enough to hold them, caused a number of cases of temporary overexposure. The worst example of this was the storage of a dental compound applicator, containing 100 mg. of radium and used for a few hours daily, in a small enameled basin in the medicine cabinet of the nurses' office. Nurses sat within 3 feet of this applicator when charting and stood within 1 foot of it when they prepared drugs for distribution, and they received over 0.5 r per hour of such work done. A similar situation was found where storage was in a workroom on the operating-room floor, although in this case, exposure was less because the people working in the room seldom came nearer than 4 feet to the safe. In this case, tandems for intra-uterine application were made up a day or two in advance of use, and the amount of radium to which personnel were exposed seldom exceeded 75 mg. Another hospital had an excellent lead safe but stored a permanently assembled urological applicator containing 100 mg. on a long handle

FIGURE 1.—Inadequate storage of radium in metal containers in a medicine cabinet (right hand side of lower shelf). Large quantities often were placed in the front ⅜-inch-thick lead box.

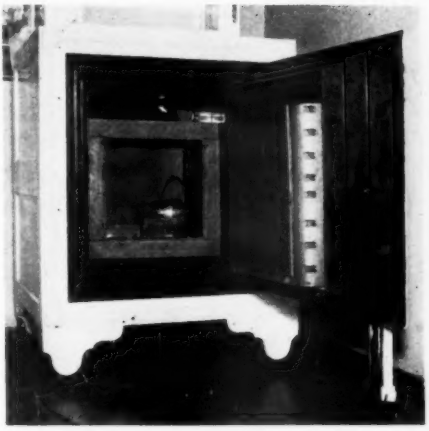

FIGURE 2.—Excellent 2-inch-thick lead-lined steel office safe. Radium units are in three smaller lead containers, 1 to 1½ inches thick.

in a ⅛-inch lead box in a steel safe near which several people worked.

An important factor in exposure was the method of distribution of units in the safe. Often all of the radium was kept in one drawer or box, and each time a portion of it was wanted there was exposure to the entire amount stored. Inner storage containers for holding all of the radium units such as cardboard pill boxes, small wooden boxes, and ⅛-inch lead boxes were found in six hospitals. These were removed and opened by hand when radium was wanted. The ideal arrangement is one in which the radium units are divided on the basis of unit size and are placed in drawers which are well-marked and have thick lead facings to protect the hands. This permits the person assembling the applicator to take out only those units he needs without exposure to the entire amount stored.

Figure 1 shows an example of storage in containers providing inadequate protection. Nurses worked within a few feet of this cabinet. Figure 2 is an example of adequate storage.

Assembly

Exposure during assembly is related to both equipment and techniques. The Advisory Committee on X-ray and Radium Protection has recommended (*12*) that preparation of radium applicators and similar operations should be done behind a lead L-block of a minimum thickness of 2 inches (5 cm.). The authors found that 14 hospitals in 45 had no L-blocks. Of those remaining, all had blocks; however, 4 were from 1/16 to 1/4 inch thick, 6 were from 1 to 2 inches thick, and 21 were 2 or more inches thick.

Although 21 of the blocks were thick enough, several of them were too short or too narrow to give adequate protection to the worker's body. In other cases they lacked either lead glass visors or wooden, linoleum, or other covering of low atomic number to reduce secondary radiation.

In a number of institutions visited, radium was assembled in several places in the same hospital. Often there was ample provision for assembly of applicators in one place, usually the radiological department; but there was no provision for assembly in the other, an operating room, where applicators were assembled with the fingers or with very short instruments and without the use of an L-block.

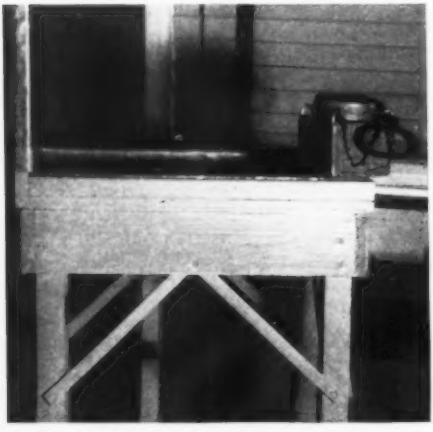

Figure 3.—Inadequate L-block made by covering a wooden frame with 1/8-inch-thick sheet lead. An excellent hand carrier is shown.

Figure 4.—Excellent radium storage and workroom. Lead L-block 2 inches thick, covered with linoleum and provided with a lead glass visor. Also shown are a lead-lined safe with radium units in individual drawers, small lead wells behind L-block, radon implant sorting boxes (white), and wheeled carrier.

Figure 3 shows an inadequate L-block. Figure 4 shows an L-block 2 inches thick.

Proper instruments for handling radium tended to be either very good or lacking. In 24 cases they were excellent, in 3 intermediate, and in 18 inadequate. Many operators used 4-inch hemostats and thumb forceps instead of the recommended 10-to-12-inch forceps. In fact, the authors noted a frequent tendency on the part of many to use their fingers instead of instruments. One radiologist characteristically began all assemblies with forceps but ended them with his fingers. As a rule, the institutions in which 4-inch forceps or fingers were used had few instruments. Those with adequate forceps tended to have a full complement of instruments.

The average person assembling applicators is not adept at this work. As a rule, applicator assembly is an occasional job, therefore no one works at it sufficiently often to develop any real skill. Assembly of applicators by a single individual varied from 4 to 100 per year, the majority being under 20. The number usually was not sufficient to enable the worker to become skillful. In only 1 instance was practice with dummy units a weekly routine for residents, internes, nurses, and technicians. Since speed and facility in handling radium at a distance are so important from a protective standpoint, more emphasis should be placed on practice with dummy units as a means of developing and keeping up one's skill.

The assembly of applicators is a task usually assigned to residents, internes, technicians, and nurses. These people frequently had little knowledge of the dangers of overexposure and occasionally had too little direct supervision. In six cases, the lumen of rubber tubing used for tandems was so small that considerable time was spent inserting radium units covered with lubricating jelly into it. It was found that such procedures could not be carried out without holding the rubber tubing or radium unit in the fingers or in very short forceps. In one case, 15 minutes elapsed while three 25-mg. units were placed in tubing. Another cause of considerable exposure was the fact that there was no provision for holding the tubing during insertion of the radium units and tying even though it had an adequate lumen. Where there was a vise on the block, it was possible to keep the hands at some distance from the radium during most operations; but only 2 in 25 institutions where rubber tubing or fountain-pen sacs were used, provided such a means for holding applicators. Metal applicators made in sections which could be screwed together to give a variety of lengths and shapes were used for intra-uterine therapy in 20 institutions, and these were handled with considerably more speed and less exposure than were the rubber tubes.

A few examples of exposure measurements made during assembly of tandems for intra-uterine insertion under varying circumstances are given in table 2. Measurements on the body were made by having the person doing the assembly carry a 0.25-r, full-scale, ionization chamber in a side trouser pocket or lower uniform pocket. Those on the fingers were calculated by extrapolation, by using measurements made with a chamber taped to the wrist and the inverse square law.

TABLE 2.—*Body and finger exposures in selected cases during assembly of radium tandems for intra-uterine insertion*

Case	Applicator material	Radium		L-block thickness	Forceps length	Elapsed time	Exposure in r	
		In applicator	Other on table				Body	Fingers
		Mg.	*Mg.*	*In.*	*In.*	*Min.*		
A	Rubber	85	[1] 275	2	10	31	0.02	25
B	Silver	90	10	¾	8	12	.04	.4
C	Rubber	75	None	None	10	10	.06	1.8
D	do	75	[1] 125	None	4	20	.08	36

[1] In open lead box.

In case A the radiation to the body behind the 2-inch L-block was low, even though there was exposure to a considerable amount of radium for a long time. However, the fingers received a considerable dose because the operator had difficulty inserting the radium units into the rubber tubing. It required a long time to assemble this applicator, and the fingers were near the excess quantity of radium during much of the procedure. In case B the body exposure was twice that in A in spite of the smaller total amount of radium and the shorter exposure time, because the ¾-inch L-block offered less protection than

did the 2-inch one. The exposure to the fingers was small because the radium units were placed in the silver applicator with facility and because it was not necessary to place them near the radium, as might have been the case had a rubber tandem been prepared.

The elapsed time for assembly was short in case C, and the amount of radium to which the operator was exposed was the smallest of all cases cited. However, the fact that no L-block was provided caused exposure to the body to be considerably higher than in case B. In case D there was no L-block, and in 15 minutes body exposure was 0.08 r, just short of a tolerance dose. This technician received the highest exposure in the series of cases cited, 36 r to the fingers, because he used 4-inch forceps and frequently touched the tubing containing the radium units during tying.

These cases show that exposures of considerable size occur during assembly of a single applicator and lend weight to the recommendations that L-blocks and long forceps be used. One case also shows how these things alone are not enough and how other breaks in technique can cause high exposure. These same people received additional exposures when the applicators were disassembled, and they received more exposures in their roentgen-ray work.

Radium applicators cannot be prepared without some exposure, hence it is especially important to keep the radium at the greatest possible distance from the hands and body and to keep assembly time at a minimum. Another practice relating to the storage container was noted which sometimes led to overexposure. The storage of all radium in a single drawer or box invariably forced the workers to place the entire supply on the L-block and keep it there all the time while they worked. They often were exposed to the gamma radiation of three to six times as much radium as they needed in making an applicator.

Rotation of personnel may reduce exposure received by a given person; but, unless there is more assembly practice, exposure may be spread to a greater number of people, because each one has less chance to become adept in his work due to his infrequent handling of radium.

A certain amount of use of the fingers is unavoidable in inserting certain applicators during treatment. However, much of the overexposure noted in these hospitals during assembly is avoidable. All radiologists supervising assembly of radium applicators will find it desirable to go over each step in present procedures critically with a view to improving them from the standpoint of increasing distance and reducing handling time. Such checks should be made at frequent intervals, and technicians and nurses should not be depended upon to train new personnel unless they have been especially well trained themselves. This can be ascertained only by talking to them and watching them work and not by assuming that they know because they handled radium frequently at a previous place of employment.

The excuse was often given that it was not necessary to provide protective devices or improve techniques because so few applicators were assembled. That is a poor excuse in view of the paucity of knowledge on the threshold of exposure which causes injury. Furthermore, there is always a chance that the volume of work in a given institution may increase or that the person who has failed to learn good practice in one institution may change his place of employment and may handle radium more frequently in the next hospital. Responsibility for the storage and handling of radium should be fixed in a person who understands the physics of radiation and who has the energy and

time to check the work under his supervision at frequent intervals instead of trusting that persons once told how to do things will continue to do them correctly. Hospitals or individuals interested in providing radium therapy should provide adequate storage containers, carriers, and instruments. They should, in addition, set aside a suitable space for radium handling. It was found that failure to do this in many of the hospitals visited was a result of ignorance of the dangers of exposure and of methods of their avoidance, rather than of a desire to save money in the purchase of equipment.

TRANSPORTATION

Overexposure may occur during transportation of radium in a hospital; but even though relatively little time elapses during this process, care must be taken to hold exposure during transportation to a minimum so that it will not add an additional avoidable exposure increment to the unavoidable exposures which occur during assembly and use. Because the time involved in handling carriers containing radium is short and because they are heavy, recommended hand-carrier thicknesses are usually ½ inch or more of lead for quantities of radium up to 100 mg. When larger amounts are to be carried, thicker transportation devices should be provided, and it is usually necessary to put wheels on such carriers because of their weight.

FIGURE 5.—Small glass bottle used for carrying radium.

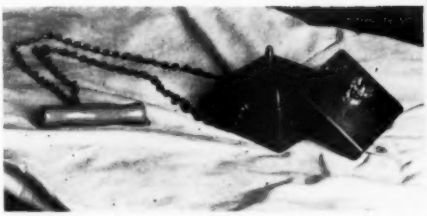

FIGURE 6.—Excellent hand carrier. Lead ¾ inch thick.

In 21 institutions there were no carriers or they were ⅛ inch or less in thickness. Five had carriers ⅛ to ½ inch thick, and the other 19 had carriers ½ inch or more in thickness. However, the fact that an institution had an adequate carrier did not mean that this device was used. In 5, excellent carriers were provided, but because it was easier not to carry them and because no one had directed that radium should always be transported in them, they were unused.

A nurse and radiologist in one institution carried applicators in their hands; and at another, for lack of a carrier, a resident carried applicators in his pocket. In several instances radium was transported in towels or carried by threads intended for use in anchoring the applicator after insertion. In a few cases the applicators were carried to and from the place of use by thumb forceps.

Location of the place of storage and assembly often were not given enough attention from the standpoint of time and distance involved in transportation of the radium between them. Storage in the main office safe, assembly at a distant place in the hospital, and use in another place,

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FIGURE 7.—Radium carrier on wheels with minimum lead thickness 1½ inches.

usually an upper floor major operating room, was a situation encountered several times. An effort should be made to bring the place of storage, assembly, and use as close to each other as possible. When the radium must be used in a number of widely scattered places in the hospital, it is best to have the place of storage and assembly nearest that location where it is applied most frequently.

Figure 5 shows a bottle used as a carrier in one of the institutions visited. Figure 6 shows an excellent hand carrier. Figure 7 shows a heavier carrier mounted on casters for easy transportation.

ROENTGEN RAY

THERAPY PROTECTION

Thirty-nine of the forty-five hospitals visited had roentgen-ray therapy equipment, and in these the type, thickness, and location of shielding with reference to personnel were noted. Adequacy of shielding in lead or lead equivalent thickness was judged on the basis of data previously published by Taylor (*6*). The following tabulation gives the lead equivalents recommended:

Roentgen rays generated by peak voltages not exceeding—	*Minimum lead equivalent*
75 kv.....	1.0 mm. (0.3937 in.)
100 kv.....	1.5 mm. (0.5906 in.)
125 kv.....	2.0 mm. (0.7874 in.)
150 kv.....	2.5 mm. (0.9843 in.)
175 kv.....	3.0 mm. (1.1811 in.)
200 kv.....	4.0 mm. (1.5748 in.)
250 kv.....	6.0 mm. (2.3622 in.)
300 kv.....	9.0 mm. (3.5433 in.)
350 kv.....	12.0 mm. (4.7244 in.)
400 kv.....	15.0 mm. (5.9055 in.)
500 kv.....	22.0 mm. (8.6614 in.)

There were only 4 hospitals in 39 which had inadequate shielding in walls and doors by these standards. The radiologists in them were aware of these deficiencies in shielding, and 3 of these institutions had taken steps which would lead to improving their therapy-room protection before our visit.

Measurements made showed an average daily exposure of 0.005 r per working day in the 35 adequately protected installations. In many of these, the readings were so small that they could not be interpreted. The maximum exposure found in them was 0.01 r per working day. In the 4 hospitals with inadequate protection, exposures were slightly over 0.1 r, the tolerance dose, per working day when the volume of cases treated was high.

Lead glass observation windows were adequate in lead equivalent in all but 2 of the 39 hospitals.

In spite of adequate shielding, practices were noted which led to overexposure. One technician entered the deep therapy room to talk with the patients or to reposition them after they moved without turning off the high voltage on the tube. It was not possible to ascertain how often this occurred, but during our visit it was done spontaneously several times, and an ionization chamber in the technician's pocket showed that she received 0.75 r during her stay in the room. A technician in another hospital sat with or held many foreign-language-speaking patients during superficial therapy. She did this on an average of three times weekly and received an average of 1.5 r per treatment. Her daily average on a 5-day basis was approximately 1.0 r.

A number of other improper practices leading to overexposure were noted, but in the majority of hospitals practices were excellent. The radiologists in charge in the institutions where overexposure of roentgen-ray therapy technicians occurred had not been checking the work of their technicians and were unaware that these breaks in technique occurred. Close and careful supervision is important.

Therapy Apparatus Calibration

Calibrations of deep therapy apparatus were made in 37 hospitals, and these were compared with the previous calibrations in use as standards for therapy at the time of our visit. In 9 institutions, or approximately one-fourth of the group, calibration differences exceeded 10 percent, ranging as high as 50 percent. The yields were lower than the hospital calibration sheets showed them to be in 7 of the 9 cases.

An effort was made to learn the reasons for these differences, and the four principal ones are as follows: (1) Calibrations were too infrequent; (2) persons calibrating were not thoroughly familiar with the proper methods of doing such work; (3) calibration equipment was not standardized; and (4) stop watches, timers, and integrating devices gave erroneous readings. The first two were the principal causes of variations.

Table 3 shows the distribution of calibration differences over 10 percent with reference to frequency of calibration. In one hospital the radiologist did a check calibration every morning before starting treatment. The opposite extreme was a hospital in which the last calibration had been done approximately 3 years before our visit, during which period a new tube had been installed without calibration. It is of interest that, in the 20 hospitals calibrating oftener than once a year, only 3 showed significant errors as compared with 6 in the group of 17 hospitals calibrating once a year or less often.

Table 3.—*Distribution of calibration differences with reference to frequency of calibration*

Calibration frequency	Hospitals	Hospitals with differences greater than 10 percent	Calibration differences
	Number	*Number*	*Percent*
2 or more per month	1	0	0
1 per month	9	3	10, 12, 15
2 per year	10	0	0
1 per year	11	4	15,19,46,50
1 per 2 years or more	6	2	15, 19

Time and the ensuing tube and equipment changes are not the only factors causing errors. The method and individual calibrating are also important. In one case, one of the authors had an opportunity to calibrate a tube a few minutes after it was installed and calibrated by someone else, and found that a minus 46-percent error had been made.

With the thought that it might throw light on the personal factor, the cases in which the authors' calibrations differed from those in use were arrayed against the profession of the persons calibrating. The results are given in table 4. The fact that we agreed in all 11 cases with all the registered physicists and with all but one of the radiologists' calibrations led the authors to believe that their own calibrations were accurate within the limits of experimental error. As a rule the institutions in which physicists and radiologists calibrated had such checks oftener than once a year. Calibrations were done by persons in these groups in 27 of the hospitals and were accurate in all except one. In the remaining 10 institutions in which technicians or others calibrated, 8 showed errors over 10 percent. From these findings it would appear that calibrations done by radiologists and physicists are the most likely to be accurate, and that persons doing such work should have more than a casual knowledge of the physics of radiation.

TABLE 4.—*Calibration differences of over 10 percent, by profession of worker calibrating*

Profession of calibrator	Institutions in which calibrators worked	Errors over 10 percent
	Number	*Number*
Registered physicists	11	0
Radiologists and staff physicists [1]	16	1
Technicians	3	2
Others	7	6

[1] Includes residents in radiology.

The calibration errors in two instances resulted from measuring radiation intensity with the ionization chamber and electrometer connected during measurements with no cones, or with large cones which permitted the direct beam to strike the electrometer. The lead shielding of the electrometer is not sufficient to prevent errors of considerable magnitude in such cases. Only the ionization chamber should be placed in the direct beam of roentgen rays, as is recommended by the manufacturer. Uniformly accurate measurements are possible if this method is used and if the electrometer fiber is adjusted back to zero before readings are made.

The calibration devices should be restandardized at least once a year. Many of the calibration variations found occurred in institutions in which calibration equipment had not been standardized for many years. Often in areas where average humidity is high, calcium chloride in the drying chamber was completely saturated with water making it difficult to charge the instrument. In other cases the equipment used was borrowed, and the user had no assurance that the instrument was accurate.

Calibration should be done only by properly trained and experienced personnel. It frequently was found that hospitals required that the person selling or installing new therapy units and tubes calibrate them. Occasionally these persons were not qualified to do such calibrations or did not possess standardized calibration equipment. It is understood that manufacturers discourage such practices.

Stop watches and timers used for intensity measurements are important, and a quick-acting, remotely controlled shutter greatly simplifies measurements. Several calibration errors, most of them

under 10 percent, were found to be due to such defects.

Some radiologists had difficulty in obtaining consistent readings because of the high sensitivity of their chambers. If this sensitivity is so great that only a few seconds are used to obtain a reading, "end effects" become very important. A 15-second-exposure interval is all that is usually possible when a 25-r chamber is used at the conventional treatment distance, and errors as small as 2 seconds in timers or integrating devices become important. The use of a 100-r chamber enables one to operate for a full minute or more, and a few seconds error is then less important.

Line voltage fluctuations occurred in many hospitals and were excessive in four. In one, the range was from a low of 18 milliamperes to a high of 27 milliamperes.

Therapy apparatus was relatively new and was of shock-proof design in most of the hospitals visited. Only two installations presented shock hazard.

Roentgenoscopy

The widespread and frequent use of roentgenoscopic apparatus has made the problem of exposure during its operation an important one. The situation is complicated by the fact that workers must be in the room with the equipment and cannot work in a completely shielded environment as they can in roentgenography and therapy.

Direct radiation is prevented from striking the operator by the use of lead or lead-containing devices such as tube housings, cones, shutters, and glass in screen holders as part of the roentgenoscopic apparatus. In addition, the patient absorbs some of the direct radiation, and gloves containing lead are worn by the operator to protect his hands from direct and scattered radiation. The workers in the room prevent scattered radiation from reaching most parts of their bodies, especially the gonads, by wearing lead-impregnated aprons.

Ionization measurements were made on the top of the lead glass of the screen holder, and these showed that the glass provided adequate protection in the entire group of 42 units checked. However, examination with a portable roentgenoscopic screen showed that the direct beam overlapped the lead glass in 30 of the units when the screens of the tables were 40 cm. above the examination-table tops and the shutters were wide open. The overlapping varied from 1 to 6 inches on any one side. Figure 8 shows a film exposed at the edge of a screen under such conditions. Large exposures to the hands, forearms, arms, face, and chest of the radiologist are possible under such circumstances in view of

Figure 8.—Film exposed 5 seconds with shutter or radioscopic unit wide open. Note protection offered by lead glass of the screen and exposure caused by spread of primary beam beyond edges of the screen.

the fact that the average intensity was 13 r per minute in air at the table top.

Radiologists try to keep the shutter aperture as small as possible and assume that they avoid direct radiation. However, it was found that many occasionally opened the shutters to their limit, especially at the beginning of an examination.

The excursion of the shutters of most apparatus can be controlled by adjusting the shutter control stops. A study of the amount of overlapping on all sides of the screen at full aperture revealed that either the tube or the screen was off-center in many cases. The discovery that old and new equipment alike permitted spread of the beam beyond the edges of the screen in so many cases suggests that a careful check should be made to see if the adjustments are correct when equipment is installed and that it is important to check them at intervals thereafter. Our experience indicates that such mechanisms do not stay in permanent adjustment.

FIGURE 9.—Film exposed for 5 seconds on table top of radioscopic unit used for fracture reductions. Shutters were closed. Note exposure, caused by primary beam extending beyond the outer edges of the shutters.

Five hospitals had equipment with semi-fixed screens and movable tubes. In these institutions it was virtually impossible to prevent overlapping in use.

In further consideration of overlapping direct beams, the common use of obsolete roentgenoscopic apparatus and small hand-held or head-operating screens during fracture and other work represent a serious hazard, especially if those using the equipment are unfamiliar with radiation physics and the necessity for taking precautions against overexposure. Figure 9 shows a film exposed on the table top of a unit used for fracture work.

Protective gloves with a lead equivalent of 0.5 mm. are recommended as a safeguard against direct and scattered radiation, and the results of our tests of apparatus verify the need for following the recommendations of the Advisory Committee on X-ray and Radium Protection if one wishes to reduce the amount of radiation reaching the hands. In one-fourth of the institutions visited, the lead equivalents of the gloves were less than 0.5 mm., the gloves had holes in them, the sides of the fingers lacked lead rubber, or the gloves were made from pigskin or canvas and contained no lead. Many of those using pigskin and canvas gloves thought that there was no direct or secondary radiation of high voltage from which they needed to protect themselves and that these gloves would stop the soft roentgen rays present. The proportion of more penetrating radiation was much higher than they realized in almost every instance.

Scattered roentgen rays produced in the equipment were found to be excessive in many cases. Roentgenoscopic rooms were checked at various points with a hand roentgenoscopic screen, and it was found that in 27 of the rooms there was sufficient

penetration and fluorescence 6 to 10 feet from the tube to resolve the bones of the fingers when they were used as test objects. Taylor (*6*) has pointed out that any such indications of fluorescence are excessive and has recommended that they should be remedied immediately.

Aprons were used for protection in many of the institutions visited, and an effort was made to check their effectiveness. Although the wearing of aprons with a 0.5-mm. lead equivalent has been recommended, many were found to be below this standard. The protection factors in the aprons tested varied from 2 to 100 times better than no apron. In one case roentgenoscopic procedures could be done through 8 thicknesses of the apron. Adequate ones were impregnated with enough lead to prevent fluorescence when only one thickness was used.

Technicians wore aprons in only 7 of the 42 hospitals checked, in spite of the fact that overexposure could occur in 27 of them.

The arrangement of the roentgen-ray tube housing and cone were found to be important factors in the amount of stray radiation. The short cones with rapidly diverging sides and the tables which had a wide gap between the top of the cone and the table top had the highest amounts of stray radiation.

A number of radiologists had done an excellent job of augmenting the shielding around the tube by the addition of $\frac{1}{16}$-inch lead sheeting.

In 26 institutions the daily exposure received during roentgenoscopy by at least 1 worker in each exceeded 0.1 r. The extreme was 1 institution in which the equipment provided little protection, and the radiologist, who wore no apron, received 2 r per day on the body. The users assume that the equipment, especially when it is new, provides protection (although no such claim has been made for it), and the manufacturers assume that the users wear good protective aprons, hence each group contributes unintentionally to overexposure. New designs undoubtedly will offer greater protection in the future, but the worker must still be burdened by his apron.

Radiography

The problem of providing protection to the radiographic staff is a varied one, since the demands upon the roentgen-ray equipment are never constant. The energy, currents, and exposure times required cannot be indicated by a single set of values, but depend in each case upon the physical nature of the problem. Unlike fluoroscopy, there is not the need for the presence of personnel in the room during most radiographic work. Thus the shielding of the technician operating the instrument is the major problem.

Twenty-three of the forty-five hospitals had excellent lead-lined control rooms equipped with lead glass windows. Three of the remaining twenty-two had partly shielded rooms with either insufficient lead or missing doors; 6 had portable lead-covered screens approximately 3 x 6 feet in size behind which the technician stood; and 13 had no protection of any kind, except that around the tube which was usually incomplete and insufficient.

The provision of well-lined control rooms was not sufficient to assure protection, because the tendency on the part of technicians to leave doors open was found to be frequent. In three institutions, the equipment was so installed that the direct beam was pointed toward an open or missing door whenever films were exposed on patients in the upright position. One of these departments was a busy one and used the apparatus in question for doing sinus series. The average day's sinus

examinations alone permitted those at he control panel to receive 0.2 r.

Many technicians held babies and for eign-language-speaking patients during radiography and received sizeable exposures. One technician averaged 2.0 r on her hands per day while holding patients. Another received 35 r holding one patient. Often the hands and body were in the direct beam when the technician stood against the table while working. The exposure to the direct beam was worse in some hospitals than in others, because restricting cones were not used on the tube heads. In many hospitals parents, nurses, and orderlies held patients whenever it was necessary, thus rotating exposure and preventing the technician from being overexposed. As a rule, the excuse given in hospitals in which there was no rotation of personnel was that there were insufficient people accompanying patients to enable the technicians to avoid holding them. In some institutions most children were held mechanically; while in others they were held by hand, and every film showed the holder's hands as well as the part of the patient exposed.

Many open tube bowls were in use in the hospitals visited, and few of these had been modified to cover the open top or reduce shock hazard. Mutscheller (*13*) reported measurements of radiation intensities around such apparatus apertures at 80 kv. and 10 milliamperes, and found intensities of 1.06×10^{-3} r per second at 80 cm. on the open side away from the target. Intensities at 50 cm. on the side protected by the lead glass bowl were 1.7×10^{-5} r per second. He found intensities greater than 4.0×10^{-4} r per second at 70 cm. on the sides directly opposite the tube ends where the lead glass bowl is cut out to permit the tube ends to project.

Technicians often were unaware of the dangers of standing opposite the open side of the bowl with the tube pointed laterally during radiographic work.

Radiation coming through the top of open tube bowls can be eliminated by the addition of a cover similar to the one described by Taft (*14*). Additional lead shielding should be provided to cover the region of the tube ends, and shockproofing should be carried out. One might question the desirability of bothering to make such alterations since, at best, the equipment will still be makeshift. It is preferable to replace such tubes and bowls by newer, modern equipment whenever possible.

A few technicians whose chief work was bedside radiography in large hospitals received considerably more than 0.1 r per day to their bodies. Only one instance was noted in which the technician wore a lead rubber apron during bedside work. A number of cases were noted in which technicians held cassettes upright during lateral exposures. In one, the exposure of two plates gave 0.42 r on the hand holding the cassette and 0.14 r on the body.

Injury

Extensive study of injuries of workers was not feasible in this survey owing to time limitations. However, a few observations were made.

Blood counts were routine procedures in only 9 institutions. The time elapsing between these counts varied from a month to a year. Technicians and radiologists in the remaining 36 hospitals usually had no counts, although occasionally a technician who felt weak and tired had a count done on his own initiative. Several individuals in the group having routine counts had total white counts under 5,000. Several had low total red counts and low hemoglobin. One technician had developed a persistent leukopenia. This worker

had been employed in one of the institutions where high exposures were found and showed no evidence of recovery after 2 months' rest from her association with radiation. As a rule, however, the hospital laboratory counts were not planned and done on an objective basis, and evaluation of changes in them and their relationship to radiation exposure cannot be made. One physician reported having had mononucleosis 13 years earlier, and another reported a 10-percent eosinophilia 3 years before our visit. Both had recovered and attributed their blood changes to earlier heavy exposure to radiation. However, this may be open to question.

Skin injuries were observed in 17 individuals, of whom 12 were radiologists and 5 were technicians. These changes varied through the scale of classical injuries from atrophy to keratoses in all except 2, who had epitheliomas. One of these also had bone destruction which necessitated amputation of her phalanges.

The individuals with these injuries had worked with radiation for periods ranging approximately from 10 months to 25 years at the time of our visit. One technician showed skin changes on her fingers after 10 months of work during which she held an average of 5 patients daily during radiographic procedures. Another had damage after a year during which time it was her custom to hold dental films in patients' mouths. All of the remaining group with skin injuries had worked with radiation over 7 years, and the majority had worked 14 years or more.

Approximately 225 people came under observation during the study, three-quarters of whom were technicians. The ratio of skin damage in technicians to that in radiologists was low in the hospitals visited for two principal reasons: (1) They were not exposed to primary radiation as often as were the physicians doing roentgenoscopy and handling radium; and (2) their average experience on the basis of years of exposure was low as compared with that of the group of radiologists and their assistants in these hospitals.

No histories of abnormal offspring were elicited, although several possible cases of sterility were noted. The incidence of genetic injury could not be evaluated, although such damage may be present in many of those seen and may not be evident for many generations to come.

DISCUSSION AND RECOMMENDATIONS

No hospital visited had provided optimal protection in every regard. It was observed, however, that most of the radiologists were becoming increasingly aware of their problems and were taking steps to reduce exposures.

In spite of the fact that the impression prevails that injuries no longer occur, the finding of skin changes in nearly one-quarter of the radiologists seen raises a question regarding the validity of this assumption. It is probable that these injuries will not prove to be as serious and deadly as were those in the early days of roentgen-ray and radium use when exposures were greater.

The skin damage seen by the authors suggests that much remains to be accomplished in preventing overexposure, and it would be interesting to see the results of a careful Nation-wide survey of injuries among radiation workers.

The work on genetic injury points to the fact that there may be no minimum tolerance dose for gene and chromosome damage. Such injuries need not manifest themselves in gross abnormalities in first-generation offspring as most persons unfamiliar with genetic mechanisms assume, but may appear in the form of lessened fertility rates owing to lethal effects or

may appear as gene mutations and chromosome aberrations leading to abnormalities of various degrees in distant generations.

In setting a standard tolerance dose it is best not to set different doses for various parts of the body until more is known about biological effects of radiation. It is desirable, from a practical standpoint, to hold exposure of all parts as low as possible. There are certain limits below which we cannot operate even with the best present-day techniques, but the authors found that many workers kept their full-body exposures well below 0.02 r per day by carefully following the recommendations set forth by the Advisory Committee on X-ray and Radium Protection (*12*, *13*).

Some people have already reduced tolerance doses in their laboratories below 0.1 r. For example, Tuve, of the Department of Terrestrial Magnetism of the Carnegie Institution, Washington, D. C., has posted a permissible laboratory tolerance dose of 0.01 r for his electrostatic generator and cyclotron workers.

The authors have learned with interest that the Advisory Committee on X-ray and Radium Protection has recently lowered its recommended tolerance dose from 0.1 r to 0.02 r.[1]

There is a tendency to neglect the mechanical devices for affording protection during radium handling, and the infrequent assembly of radium leads to slowness and lack of dexterity in handling it. More attention must be paid to formulating rules for its handling and more time must be devoted to practice assemblies, especially in the places where it is used weekly or less often. Certainly those learning to assemble applicators should use dummy units and not actual radium units as is done in most institutions. The relatively infrequent use of radium and the facts that it occupies little space and appears to be quite harmless, account for much of the lack of respect for the dangers incident to handling it. Roentgen-ray equipment, on the other hand, by virtue of its size, formidable appearance, and voltage, has encouraged more respect for the potential injury it may cause.

A consideration which frequently was found to have been overlooked in storage was the matter of location of the storage container with reference to the place of work of hospital personnel. All too often, the radium was too near office workers and technical personnel. Distance and inverse-square-law considerations, as they affect dose, are as important in protection as the provision of absorptive materials, yet it was found that little attention had been paid to them in many hospitals.

It must be emphasized that the storage block itself must have spaces large enough in it to hold the largest assembled radium applicator. Too frequently were tandem or other applicators, because of their size, left outside the block. Either these applicators should be torn down after use or sufficient storage space should be provided in the protective container for the entire applicator.

Measurements have shown that a large amount of exposure is caused by the practice of placing the entire amount of radium available on the assembly block during manipulation. Careful distribution of the radium units in the storage container in separate drawers, the labeling of each drawer, and the use of a protective lead "well" on the assembly table will greatly reduce exposure.

The use of administration-office safes for storage of radium often creates a hazard other than those which may be created by the location and thickness of the storage container. Radium applicators occasion-

[1] *Information from Lauriston S. Taylor, chairman, Advisory Committee on X-Ray and Radium Protection.*

ally were removed from patients at night, and since the administrative staff was not on duty, the radium often was temporarily stored in desks, drawers, and very thin lead containers. Provision must be made so that access to the proper storage block is always possible. Supervision of these procedures must be constant and placed in the hands of someone adequately trained in the hazards of handling radium. Radium should never be transported in cardboard boxes, glass bottles, bare hands, or pockets as was found in this study. Lead carriers on long handles or on wheels should always be used.

Education of all radiation personnel on protection practices should be stressed as much as the provision of protective devices. This education demands supervision, and it should be the policy of the supervisor that no untrained personnel should ever receive any exposure either directly by handling radium or indirectly by misuse by others. Much overexposure in the manipulation of radium was due to lack of supervision of technicians.

It has been pointed out that exposure during the use of roentgen-ray equipment for therapy was found when the operators entered the treatment room with the tube still in operation. This is in direct violation of all existing recommendations and should never occur. Deep roentgen-ray therapy equipment has been well-protected by shielding, but the large number of calibration errors found indicate that such measurements must be done frequently by competent persons with standardized equipment. The radiologists and registered physicists were found to be the most competent groups calibrating equipment. In view of the accuracy of measurements made by registered physicists as shown by the check calibrations made by the authors, it is recommended that greater use be made of this group of workers in the calibration of equipment when the radiologists are too busy to spend the time necessary to make measurements. Whenever possible, the radiation laboratory will find it valuable to have a radiation physicist as part of its staff or as a consultant.

Roentgenoscopy is one of the worst offenders in causing overexposure. Adequate protective aprons and gloves must be worn by radiologists if exposures are to be kept at a minimum with present-day equipment, and technicians and others present during examinations also must wear aprons in most instances unless scattered radiation values are known to be low. Manufacturers must recognize the need for building the maximum protection into equipment and must not depend on the user's wearing gloves and apron. Much more protection can be built into equipment without greatly increasing cost. A sliding cone device which will bridge the wide gap between the present upper edge of the short cone above the tube and the table top might be devised which would automatically rise into place when the plate holder is rolled aside. This would add materially to increased protection from scattered radiation. More use could be made of movable lead rubber screens either on chairs or on pulleys hung from the ceiling.

So many gloves and aprons were found to provide inadequate lead-equivalent protection that it seems desirable to have some agency or council test and approve protective devices on a voluntary basis much as the Council on Physical Therapy of the American Medical Association now tests and approves. Manufacturers should be encouraged to submit their products for testing, and radiologists and hospitals should never purchase devices which do not show their protection equivalents when new.

A group of radiologists and surgeons

might well study present practices in the use of roentgenoscopic equipment in fracture reduction. More information should be provided for the orthopedic surgeon using such equipment so that he will have a clearer understanding of radiation physics and the protection he should provide for himself and his assistants as well as his patients. The practice of giving orthopedic surgeons the old, obsolete, roentgenoscopic equipment should be discouraged.

Most radiographic practices are good, but more attention must be paid to the holding of patients. Hospital superintendents and nursing supervisors must be prevailed upon to send floor nurses or orderlies with patients who must be held so that proper rotation can be carried out and so that the roentgen-ray technicians do not receive all of the exposure. The old open bowls should be replaced at the first opportunity. Hospitals must provide, plan, and construct more adequate quarters for radiological departments in many cases and should not just put them in spare basement rooms not properly designed for such equipment and where at best, protective measures will be makeshift.

The first case of injury resulting from use of the relatively new photoroentgenographic equipment has been recorded by Reinhard (*15*). The protection practices to be followed in the use of such equipment with reference to lead barriers and lead-lined control rooms for the operators should be studied carefully. Such equipment is finding increased use in the screening of large groups of people in tuberculosis surveys by health departments, hospitals, and recently, by draftboard and induction examiners as a part of the selective-service program.

The rush of examining and treating patients has kept most radiologists busy, but they will find it desirable not to overlook matters which may have seemed minor in the past. They should spend considerable time in educating technical assistants and in checking them as they work.

Dental films have been used by many as a check on overexposure. This method is a crude one at best and has been found to have given a false sense of security in a few instances. More exact methods for making measurements should be used, and film-exposure methods should be considered only when it is found to be impossible to obtain equipment for making more exact studies.

Electrometers now in use can be utilized in making exposure measurements if sufficiently sensitive ionization chambers are provided, and new relatively inexpensive devices specifically designed for making measurements of small amounts of radiation are available. Radiologists and physicists should make exposure measurements at intervals in their laboratories. Safety directors and other responsible persons in industries using roentgen-ray apparatus or radium for testing purposes also should make periodic exposure measurements and should take adequate steps to hold exposures at the minimum possible level.

As National Defense activities increase, many technical personnel are being shifted to new positions or are being drafted, and industry is utilizing new radiation methods demanding additional equipment and personnel. The new activities may cause still further overexposure. It is urged, therefore, that protection surveys and educational programs include industries and defense activities.

REFERENCES

(1) MARCUSE, W.: Dermatitis und Alopecie nach Durchleuchtungsversuchen mit Röntgenstrahlen. Deutsche med. Wchnschr., 22: 481–483 (1896).

(2) COLWELL, H. A., and RUSS, S.: X-ray and radium injuries; prevention and treatment. Oxford M. Pubs. New York and London (1934).

(3) NIELSON, J.: Chronic occupational ray poisoning. Acta radiol., 13: 385–386 (1932).

(4) FURTH, J., and FURTH, O. B.: Neoplastic diseases produced in mice by general irradiation with X-rays. Am. J. Cancer, 28: 54–65 (1936).

(5) HENSHAW, P. S.: Biologic significance of the tolerance dose in X-ray and radium protection. J. Nat. Cancer Inst., 1: 789–805 (1941).

(6) TAYLOR, L. S.: X-ray protection. J. A. M. A., 116: 136–140 (1941).

(7) PACK, G. T.: Treatment of cancer and allied diseases. v. 1, pp. 274–300. Paul B. Hoeber, Inc., New York (1940).

(8) FAILLA, G.: Radium protection. Radiology, 19: 12–21 (1932).

(9) KAYE, G. W. C.: The protection of radium workers from gamma radiation. Brit. J. Radiol., 8: 6–26 (1935).

(10) QUIMBY, E.: Radium protection. J. Appl. Phys., 10: 598–608 (1939).

(11) NATIONAL BUREAU OF STANDARDS: Handbook HB 20, X-ray protection. U. S. Government Printing Off., Washington (1936).

(12) NATIONAL BUREAU OF STANDARDS: Handbook HB 23, Radium protection. U. S. Government Printing Off., Washington (1938).

(13) MUTSCHELLER, A.: More on X-ray protection standards. Radiology, 22: 739–747 (1934).

(14) TAFT, R. B.: Protective cover for tube bowl. Am. J. Roentgenol., 28: 562 (1932).

(15) REINHARD, M. C.: X-ray protection. Staff Bull., N. Y. State Dept. of Health, 22 (6): (March 15, 1941).

Biologic Significance of the Tolerance Dose in X-Ray and Radium Protection

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One-tenth to two-tenths roentgen per day has become generally accepted as the daily intensity of X-rays or gamma rays a human being can safely withstand.[1] The acceptance of this value has a most interesting, though somewhat peculiar background, and the standard has been made to apply to only part of the injuries produced in humans. The purpose of this report, therefore, is to review carefully the history of the present safety standard, to list the important types of injury now recognized, and in particular to show how the present concepts of protection cannot be applied to a certain class of injuries. The paper by Cowie and Scheele (*5*) indicates clearly that questions on protection are continually arising among practicing radiologists and radiation workers for which accepted answers are not available. The lack of answers is owing in part, it seems, to insufficient basic information and in part to failure to consider information now at hand.

HISTORY OF THE TOLERANCE DOSE

Serious irradiation injury has been suffered in humans since the very advent of X-rays. As early as July 1896, only a few months after the discovery of X-rays by Roentgen (November 1895), a severe case of X-ray dermatitis was described by Marcuse (*6*). Injuries of a lesser nature (soreness of the eyelids and depilatory effects) were reported even before this (*7*, *8*). The great enthusiasm which accompanied the observation of bones and internal organs by fluoroscopic means caused many persons to subject their bodies to prolonged exposure to the new radiations. Owing presumably to the low penetrating power of the radiations then available, most of the injuries were confined to the skin—especially of the hands. In 1902 the first X-ray-induced carcinoma was reported by Frieben (*9*). This occurred in a physician some months after the first appearance of an X-ray dermatitis. As a result of injury sustained already by X-ray workers, the number of X-ray tumors increased rapidly during the next few years despite the fact that numerous warning articles were published; and by 1911 Hesse (*10*) was able to collect 94 cases, 50 of which occurred in radiologists. In 1904 Milchner and Mosse (*11*) reported degeneration of white blood cells (of the rabbit) both of the lymphoid and myeloid series, and in 1905 Heineke (*12*, *13*) demonstrated the remarkable sensitivity of lymphoid organs of animals to X-rays.

Injury to the reproductive organs resulting in sterility was noted in rabbits and guinea pigs as early as 1903 by Albers-Schönberg (*14*) and in 1904 by Bergonié and Tribondeau (*15*). This, in fact, is the work which led to the generalization on radiosensitivity of cells that is now called the law of Bergonié and Tribondeau. Permanent and temporary sterility in humans has been much discussed since those early experiments, and a considerable literature has accumulated on the

[1] *Recommendations of the International X-ray and Radium Protection Commission, 1934* (1), *1937* (2), *and the reports of the U. S. Advisory Committee on X-ray and Radium Protection, 1931* (3), *1936* (4).

subject—mostly in later years, as one may observe by referring to the monograph by Colwell and Russ (*16*) (1934). Bone necrosis following severe X-ray dermatitis of the hand was noted in 1899 by Walsh (*17*). Experiments on animals in 1903 by Perthes (*18*) demonstrated inhibitory effects on bone growth, and extensive osteogenic sarcoma resulting from gamma radiation in watch-dial painters was described by Martland (*19*) in 1931. Diseases of the lung including tumors have been an industrial hazard among miners in certain localities (Bohemia and Saxony) for centuries, but it was not until 1926 that radon in the atmosphere was suspected as being one of the causative agents producing the tumors (*16*, *20*, *21*). Post-conception irradiation injury to offspring resulting in monsters and deformities must also be mentioned as one of the ill effects caused by radiation, particularly in the case of therapeutic doses (Zappert (*22*) in 1925; Hickey and Hall (*23*) in 1927; Murphy (*24*, *25*) in 1929; and Abels (*26*)). Thus, while other deleterious effects have been noted, such as injury to vascular endothelium, glandular disfunction, radiation nephritis, meningomyelitis, and the like, enough have been mentioned to indicate that a considerable variety of injuries were recognized early in irradiation history.

It is of interest also that as early as 1902 attention was given to the question of how much radiation a radium or X-ray worker might withstand. At that time the suggestion was made by Rollins (*27*) that "if a photographic plate is not fogged in seven minutes, the radiation is not of harmful intensity." Although it was known within a few months after the discovery of X-rays that this radiation is more effectively stopped by lead than most other materials and as early as 1903 that scattered radiation existed (Taylor (*7*)), there is no record of further work on protection for a period of 13 years despite the fact that cases of injury continued to accumulate. This was due largely, it seems, to the fact that those who had the privilege of working with X-rays and radium were so much impressed with the new opportunities for research and advancement of human welfare that they refused to give attention to matters of personal injury.

What is believed to be the first organized step toward protection from roentgen rays was taken in 1915 following the reading of a paper on protective devices before the British Roentgen Society (Russ (*28*)). At that time a resolution was passed providing that the council of this society should meet and take steps toward securing safety for X-ray operators. Because of the war activity which existed then, this plan failed to bring forth important advancements. As a result of the war demands, caution gave way to action, and protection measures were again forgotten. The taking of increased risks at this time probably was a factor which contributed to an unfortunate development in 1919–21, both in this country and in Europe when a number of prominent radiation workers died of apparent irradiation injury, particularly aplastic anemia. Unfavorable publicity developed, and definite action resulted.

While some steps toward protection were taken in France immediately after the war, the first roentgen-ray protection committee was formed by the American Roentgen Ray Society in September 1920. Following the publication of certain terse comments in the London Times in March 1921, concerning the sad plight of X-ray workers, the British X-ray and Radium Protection Committee was formed. The British committee (*29*) presented its first recommendations in July 1921, and the American committee (*30*) made some suggestions for protection in September 1922. The two sets of recommendations

were much the same in substance, protection being dealt with chiefly from the standpoint of absorptive screening and remoteness (meaning significant distance). Taylor (*7*) gives a vivid account of the early history of X-ray and radium protection, in his chapter in The Science of Radiology. Later general developments are described by Leddy (*8*) (1940), Kaye (*31*) (1940), and Taylor (*32*) (1941).

From the standpoint of safety, it is interesting that the early committees (1920–21) recognized merely "visible injuries to the superficial tissues, derangements of internal organs and changes in the blood" as the injurious effects to be guarded against (*29*). While "derangements of internal organs" is indefinite, it is known that damage to the reproductive organs was an effect about which there was considerable concern.

The next important development was an attempt to ascertain some estimate of the limit to which an individual could be exposed to radiation without sustaining noticeable injury—in other words, to find the "tolerance dose." The procedure followed by a number of investigators was to formulate some kind of quantitative evaluation of the amount of radiation (usually X-rays) reaching persons who had worked with the agent for a number of years and remained in good health. The results obtained are shown in table 1. Up to and including 1928, the tolerance doses were expressed in terms of the erythema dose but this can readily be changed to roentgens by multiplying by 600, the number of roentgens of 200-kv. X-rays required to produce a mild erythema. It should be mentioned also that the results were based on a very small number of individuals in each case, from two to "a very limited number" (*33*), thus causing the findings to have limited reliability.

TABLE 1.—*The tolerance intensities recommended by different authors*

Author	Date	Erythema per month	r/day
Mutscheller (*33*)	1925	0.01	0.2
Sievert (*34*)	1925	.01	.2
Glocker and Kaupp (*35*)	1925	.01	.2
Solomon (*36*)	1926	.1	2.0
Dutch Board of Health (*37*)	1927	.002	.04
Barclay and Cox (*38*)	1928	.0084	.168
Bouwers and Van der Tuuk (*39*)	1930	.01	.2
Failla (*40*)	1932	.001	.02

Kaye (*41*) in 1928 referred to the works of Mutscheller, Sievert, Solomon, Dutch Board of Health, and Barclay and Cox and adopted 0.12 r/day (1/1000 erythema dose in 5 days) as an intermediate value. In 1928 the question of protection from X-rays and gamma rays came before the Second International Congress of Radiology for consideration and action. In view of the fact that the recommendations in use in England were adopted by the Congress almost *in toto*, it is clear that Kaye's tabulation on tolerance dose must have influenced the steps that were taken. It should be noted, nevertheless, that the recommendations (*42*) adopted by the International X-ray and Radium Protection Commission, which were set up in 1928, contained no reference to tolerance dose, merely stating, as had been done previously in 1921, that the known effects to be guarded against are: "(a) Injuries to the superficial tissues, (b) derangements of the internal organs and changes in the blood." Likewise, the Commission's report (*43*) made in 1931 contains no statement in regard to the tolerance dose, although in the two subsequent reports (1934 (*1*) and 1937 (*2*)), this dose is tacitly stated as being 0.2 r/day.

As an outgrowth of the International Commission there was formed in the United States an Advisory Committee on X-ray and Radium Protection (Taylor (*44*)) to deal with particular problems in this

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country and draw up recommendations. This committee published its first proposals (*3*) in 1931 in which the tolerance dose was set forth as 0.2 r/day, thus being in general agreement with Kaye's estimated figure given in 1928. In a later report in 1936 which is the last (*4*), the tolerance dose is stated as 0.1 r/day, no explanation being given for the change. In a recent publication (1941) on the subject of radiation protection, Taylor (*32*), who is chairman of the American committee, referred to the safety value as being 0.02 r/day, a still smaller figure, but one which is in agreement with that recommended by Failla (*40*) in 1931. Surely the variations cited indicate an uncertainty in regard to the tolerance value and a trend toward reducing it to provide greater safety.

It is noteworthy that by far the greater amount of writing on radiation protection has been done by physicists.[2] This is fortunate because protection for the most part is provided by cutting down the amount of radiation reaching operators, a type of work on which physicists are most able to advise. It seems so obvious today that remoteness (because the inverse-square-law principle applies) is one of the most important factors in protection and that lead is one of the most effective absorbers; yet at one stage these things were not known. According to Kaye (*31*), some persons very early in the history of X-ray and radium proposed that X-rays could best be stopped by red silk or a thin layer of rubber sheeting. It is clear, however, as physicists themselves have pointed out the protection problem is by no means entirely a physical one. Kaye (1935 (*49*) and 1940 (*31*) said:

> There are, of course, a number of uncertain variables which do not lie within the province of physics, but are wholly biological, such as (1) the effect of local as against general exposure to radiation, (2) the amount of previous exposure, (3) the duration and frequency of individual exposures, (4) the intervening degree of recovery (this latter being first suggested in 1940), (5) general idiosyncrasy, and (6) selective sensitivity of particular parts of the body.

The unsettled character of the subject is further stressed by Taylor (*32*) in 1941, when he says:

> Obviously, the determination of this tolerance dose is difficult and at best uncertain. The biologic factor differs too greatly among individuals to permit the use of a sharply defined tolerance. To be well beyond the danger limit one must apply a generous factor of safety to the result of any physical measurements.

Statements of this type, made by such eminent persons in the field, emphasize strongly the need for attention to be given to the biological side of protection. Whereas answers to the questions raised by Kaye and those raised currently by radiologists are not known for all of the types of injury now recognized, there is, nevertheless, a considerable body of biologic information that can be brought to bear upon the general problem of protection, some of which will be discussed.

TYPES OF INJURY

As stated above, the early protection workers recognized only the following as irradiation changes to be guarded against: (1) Injuries to the superficial tissues; and (2) derangements of the internal organs and changes in the blood. This same wording has been used in all four of the prepared recommendations presented by the International X-Ray and Radium Protection Commission with the exception of the last (1937) where (2) was

[2] *Mutscheller* (*33*), *Glocker* (*35*), *Sievert* (*34*), *Solomon* (*36*), *Bouwers* (*39*), *Barclay* (*38*), *Kaye* (*41*), *Failla* (*40*), *Taylor* (*7*), *Braestrup* (*45*), *Evans* (*46*), *Stenstrom* (*47*), *Quimby* (*48*), *Read and Mottram* (*21*). *So far as we know there are but few exceptions* (*Read and Mottram* (*21*), *Leddy* (*8*)).

modified to read as follows: "Changes in the blood and derangements of the internal organs, particularly the generative organs." It seems strange that protection, as defined, has been made to pertain only to certain of the recognized injuries without certain explanations or inferences being made. Taylor (*32*) 1941 says:

> By the "tolerance dose" is meant the amount of X-ray energy that a person can receive continuously or at repeated intervals without suffering any damage to the blood or reproductive organs.

In this case the type of exposure has been more specifically described, but, so far as the type of injury is concerned, the description is incomplete. It seems natural that physicists have avoided making commitments on biologic matters, but it seems strange that radiologists have not shown greater concern for the types of injury to be guarded against.

From the foregoing citations and from later experiences, the following may be listed as among the more important known types of injury:

(1) X-ray dermatitis
(2) Tumor induction
(3) Sterility
(4) Leukopenia
(5) Leukemia
(6) Anemia
(7) Bone necrosis
(8) Glandular disfunction
(9) Fetal injury

More recently still another type of injury has been discussed—the genetic. Muller (*50*) in 1927 found that mutations can be produced with X-rays in the offspring of the fruit fly, *Drosophila melanogaster*. His experiment was a simple one. Adult males were exposed to radiation and then allowed to inseminate normal virgin females. Offspring obtained from this mating were examined for abnormalities which were present in a considerable portion of individuals, depending on the amount of radiation administered. The same result was obtained when ova were irradiated instead of sperm. The changes produced were of the most diverse characters, eye color, shape of wings, number of bristles, etc. Since such modifications became fixed in the germ plasm and were passed from parent to offspring through succeeding generations, they are properly termed "mutations"—in this case, X-ray-induced mutations. Such mutations have been produced in a wide variety of organisms, both plant and animal, by numerous investigators. Accordingly, we may accept as well-established the fact that X-rays produce inheritable changes in the germ plasm. Radiation-induced inheritable changes in the form of mutations may be termed "radiogenetic."

Whether X-rays produce radiogenetic changes in humans is as yet uncertain. However, in view of the fact that no other process is more universally established among different species of living forms than that of chromosomal inheritance, there appears to be no good reason for believing that such changes are not produced in humans the same as in *Drosophila*. Miscarriages and abnormal children have occurred among the offspring of radiologists and radiation workers; but only very limited attempts have been made to determine whether the proportion is higher than among persons not associated with radiation. Hickey and Hall (*23*) circulated a questionnaire for the Committee on Sex of the National Research Council requesting information as to the degree of injury produced in the offspring of radiation workers and reported their findings in 1927. Among other things, these authors stated that of the 377 radiologists investigated (usually only the father in each family coming in contact with radiation), 138,

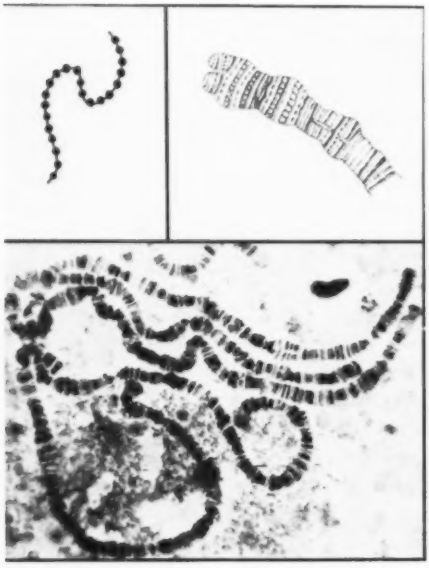

FIGURE 1.—Chromosomes. The upper left figure is a diagram suggesting the linear arrangement of the genes along the chromosome fibers. The upper right figure is a drawing of part of a *Drosophila* salivary chromosome. The lower figure is a photomicrograph of *Drosophila* salivary chromosomes.

or 36.6 percent, of the couples were sterile; that of the 262 children born to these couples before radiation employment, 2.6 percent showed some form of abnormality; whereas of the 412 born after this employment, 4.0 percent showed abnormalities; and that of the childbearing couples the average number of children per family was 2.2 as against 3.0 for physicians and surgeons living in comparable circumstances.

Murphy (*51–54*) in a series of papers in 1928 and 1930 reported the results of a study of several hundred cases of preconception and postconception irradiation. While Murphy found that approximately 10 percent of the preconception irradiated offspring were abnormal, the abnormalities appeared to him to result almost entirely from modifications produced in the mother by the radiation. Naujoks (*55*) in 1929 made a study of 91 X-ray workers and found the percentage of sterility high (24.2), the percentage of abortions about normal, and the percentage of developmental defectives rather high (4.0). These reports, preliminary as they may be, show without question that preconception irradiation injury is being produced in the offspring of humans. Whether this includes some of genetic type, however, is not clear, but judging from the results obtained with animals, including mammals (mice; cf. Snell (*56*) 1939, and Hertwig (*57*) 1939), the abnormalities may include some of this type, perhaps a considerable proportion.

Since genetic injury involves important implications and since the mechanism of genetic injury appears to be basically different from that of part, if not all, of the types of injury mentioned, the next section will be devoted to a more complete description of the mechanism of induction of genetic changes as understood at this time. As it happens, radiogenetic injury as seen in lower forms is the only one for which really definite answers can be given to the questions raised by Kaye.

THE NATURE OF RADIOGENETIC CHANGES

Hereditary components of germ cells, or for that matter any somatic cells, are known to be located as specific chemical entities in linear arrangement along the chromosomes resembling knots on a string (fig. 1). For the most part, each chemical entity, or gene, is of specific type and is required in normal ontogeny (life of the individual). Thus, not only is the presence of these entities essential, but so also is a specific linear arrangement. The genes are self-perpetuating through succeeding cell generations, and each acts at the proper place and at the proper time

during the life of the individual to exert its influence on the course of biologic activity. Hence, it is quite understandable that an external agent which would act to modify the specific organization of the chromosome would cause changes in the nature of the biologic behavior. This, in fact, is just what X-rays do to chromosomes. By some means or other (which need not concern us here) the radiation acts on chromosomes to produce the following effects (fig. 2):

(1) Gene mutation, in which the gene itself is permanently and irreversibly changed.
(2) Chromosome aberration:
  (*a*) Deletion, in which a part of a chromosome is struck loose and becomes lost through subsequent cell division.
  (*b*) Translocation, in which a chromosome part is struck loose and becomes attached to a chromosome at a new position.
  (*c*) Inversion, in which a section of a chromosome is struck loose and becomes reversed end for end.

For the most part, these effects have been observed in the fruit fly, *Drosophila*. One not closely associated with the field of radiogenetics might ask why an organism so far down the evolutionary scale is used when our main interest centers in man. The answer, however, is not far to seek. First of all, these forms can be maintained in large numbers in the laboratory with relative ease. They have comparatively short generations (about 2 weeks), thus making it possible to accumulate results rapidly. They have a great variety of characteristics such as eye color, shape of wings, number of bristles, etc., which can be followed genetically. A very important characteristic is that their chromosomes are unique in certain respects (small number, blank sex chromosome) which permit breeding analyses that otherwise would not be possible. Perhaps most important of all is that fact that *Drosophila* have giant size salivary chromosomes, permitting a correlation of chromosomal losses and deficiencies with breeding results. All of the forms of chromosome aberration mentioned have been witnessed cytologically in salivary-gland cells and correlated with visible external characteristics in many cases. It has, in fact, been possible to observe an external characteristic, such as a notched wing, and predict a deficiency that will be found at a particular location on a particular chromosome, and vice versa. It is thus apparent that a great deal is known about *Drosophila* genetics, giving one the assurance that findings with radiation are backed up with a broad background of information. For a description of this whole phenomenon

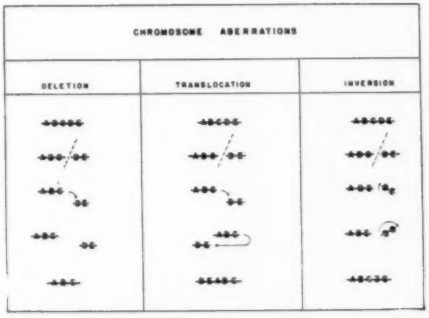

FIGURE 2.—A chart illustrating different kinds of chromosome aberration. The letters in each case represent genes located along chromosome fibers, and the dotted lines show where breaks have been produced in the fibers by radiation. In the case of deletion, chromosome parts including genes are lost; in the case of translocation, chromosome parts together with genes are relocated along the chromosome body; in the case of inversion, a chromosome part with genes becomes reversed end to end.

of genetics, the reader is referred to the recent textbook by Sinnott and Dunn (*58*). Suffice it to say here that the mechanism of inheritance and the induction of mutations appears to take place in man very much the same as in *Drospohila*. Hence, the findings in *Drosophila* may be used as a guide in the study of the conditions in man and may even serve as a basis for certain temporary measures of protection.

The visible characteristics induced by radiation through modification of the chromosomes vary over the widest range. In *Drosophila*, they may pertain to eye color, shape of wings, number of bristles, etc., as previously mentioned, but in particular to abnormalities in development resulting in death. Since genes in some way exert a great influence over the nature of development and later life, it is obvious that some of those having to do with early development are of vital importance and organisms deprived of their influence are doomed to die. Such genes are called vital, and the mutations resulting from their destruction are called lethal.

Since the changes induced in a cell by radiation are produced purely at random and lead to disorganization, it is to be expected that while disorganization is for the most part deleterious, it is likewise to be presumed that occasionally a change will be produced which is beneficial to ontogeny. In nature, through the process of natural selection and survival of the fittest, only those changes are retained which do not lead to racial destruction. In forms other than *Drosophila*, including man, the biologic changes produced by X-rays would likewise be expected to be completely of a random nature and predominantly detrimental.

Mutations are known also to occur spontaneously among cells, that is, without the aid of any known external forces. Although it is usually held that spontaneous mutation is a rare event, this opinion has rested more on the stability of species and on the low frequency with which mutants are found in nature than on data from deliberate attempts to measure the exact frequency of mutation. Muller (*59*) has devised special techniques for measuring mutation rate in *Drosophila* and has estimated that under ordinary laboratory conditions a particular mutated gene of the lethal variety may be expected in *D. melanogaster* in 0.1 to 1 percent of individuals, that is, in the chromosomes of their cells. Visible sublethal changes have been observed about one-tenth as often as the lethal variety (*60*). For purposes of illustration let us remember the value of 1 percent for the normal lethal mutation rate and 0.1 percent as the normal sublethal rate, leaving the matter of variability and the condition in humans to be discussed later.

Muller (*59*) in 1933, judging from a large amount of data obtained by himself and by others as well, estimated that the normal mutation rate is practically doubled in *Drosophila melanogaster* by a dose of 30 to 40 r applied to the germ cells lying in the gonads, an amount of radiation which is about equivalent to the doses delivered in some of the longer fluoroscopic examinations, or, as indicated by Cowie and Scheele (*5*), the amount that will reach the gonads of a good many operators at the present time in a few days to a few weeks of routine work.

Thus, judging by Muller's estimates, one might expect the probability of lethal mutation to be changed from 1 in 100 to 2 in 100 by a dose of, say 35 r, and that the chances of a visible mutation being produced would be changed from 1 in 1,000 to 2 in 1,000. From the standpoint of protection this at first might seem to show little cause for alarm, since if the

tolerance standard of 0.1 r/day is being maintained, more than a year's exposure would be required to make the chances of abnormality in offspring greater than 2 percent. Thus, with chances so much in their favor (assuming that the same conditions hold in humans), most radiation workers desiring children would be willing to take the risks involved. There are, however, certain characteristics of the genetic effect which make its dangers greater, especially when considered from a long range point of view (*60*). These, however, can best be presented after describing still other characteristics of the genetic effect and contrasting them with the characteristics of other effects.

THRESHOLD VS. NON-THRESHOLD REACTIONS

Curves may be drawn to show how the number of organisms affected in a particular way by radiation varies with the amount of radiation administered. For purposes of illustration let us consider two effects, one the induction of gene mutation and the other inactivation (killing) of tumor fragments. When a dose-effect curve is prepared for gene mutations, it is found to have the characteristics of curve *A*, figure 3 (composite results obtained by a number of investigations, which are generally consistent, are shown together by Schultz (*61*) (1936)). On the other hand, if one gives varying amounts of exposure to different samples of tumor fragments and observes these for "takes" when transplanted into animals, the number inactivated by the different doses will be found to vary as indicated by curve *B*, figure 3. Such curves have been presented by Sugiura (*62*) (1937).

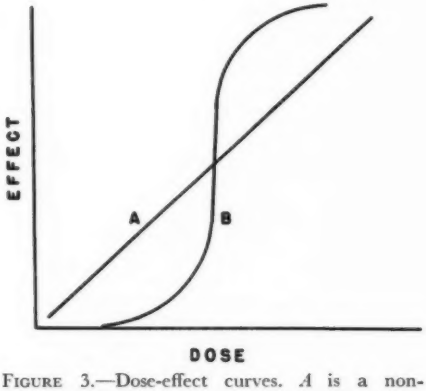

FIGURE 3.—Dose-effect curves. *A* is a non-threshold type; whereas *B* is threshold.

These curves have one outstanding difference. Curve *B* has a distinct toe or threshold character, whereas curve *A* has none. In the case of tumor fragments, it is clear that a considerable increment of dosage must be administered before any fragments are inactivated. In the case of gene mutations, on the other hand, this is different, the number of germ cells in which mutations are produced being directly proportional to the amount of radiation given. This means that as soon as exposure begins, mutations begin to be produced among the individual cells and that the mutations are produced by single random events. This is in marked contrast with tumor-fragment inactivation in which the effect is brought about only after a building up of irradiation events (the nature of which is of no interest here). The induction of mutation, therefore, involves a probability reaction, whereas the killing of tumor fragments involves one of accumulation. Said in another way, the former involves a single-event type of action whereas the latter involves a multiple-event type. Single-event action also may conveniently be termed "hit" or "target" action.

The reason why the first reaction can be said to be of single-event type and the second of multiple character is simple and for purposes of illustration let us consider an analogy. Suppose that an army is

coming over a hill and one is shooting into it at random with poison-tipped arrows. In this case, whenever a hit is registered, a soldier is killed because the injury is deadly. Killing, here, is purely a random matter. Suppose, on the other hand, several hits would be required as a rule to incapacitate a man, then some time would necessarily elapse between the beginning of shooting and the time when an appreciable number of soldiers had accumulated enough injury to be knocked out. This property of accumulation distinguishes clearly the second type of action from the first. In the second case, a soldier may escape with injury, but in the first, he either escapes with no injury or he does not escape at all.

In case of the radiologic considerations, it is clear that the proof of a single-hit killing action rests on the question of whether the curve has a threshold character, a point which is difficult to establish by experimental means. However, if the reaction is of non-threshold type certain other characteristics are automatically indicated, some of which can be investigated experimentally. Since the process of mutation induction with X-rays appears to be of single-event type, it is important to indicate the other characteristics which it should have. First, however, it should be stated that mutational changes, by definition, are irreversible (i. e., except in the case of inverse mutation) since the change becomes fixed in the cell and remains so permanently. The following would, therefore, be expected: (1) That the duration of the exposure would not affect the result—in other words, that a particular dose of X-rays given in a few seconds or in a few days to a population of individuals would produce the same proportion of mutations in each case; (2) that the effects of different treatments would be entirely additive; (3) that the effect would be directly on the chromosome; and (4) that the temperature of the specimens during treatment and the rate of metabolism would have no influence on the results obtained. These things, in fact, have all been well substantiated, particularly in *Drosophila* (cf. the following for reviews: Timofeeff-Ressovsky (*60*) 1937; Muller (*59*) 1933; Goodspeed and Uber (*63*) 1939).

Reference to mutation in this section has been limited strictly to the gene-mutation type, in order to make precise statements about the quantitative aspects of the reaction. However, with certain considerations the target hypothesis applies equally well in the case of chromosome aberrations and in this case we have the advantage of visible proof of what is happening. Sax (*64*), in a recent paper, studied X-ray-induced chromosome aberrations in *Tradescantia* and presented dose-effect curves for the changes involving single chromosome breaks in one case and double chromosome breaks in another. The first were of the characteristic non-threshold type, whereas the others were of the threshold, which is precisely what would be expected on the basis of the target hypothesis.

Thus, on the basis of the foregoing considerations, it is possible to give answers to the questions raised by Kaye as far as X-ray-induced mutations are concerned: (1) The changes result from local rather than general exposure; (2) the results of previous exposures are additive with subsequent ones;[4] (3) duration and frequency of exposures do not influence the reaction; (4) recovery does not occur; (5) general idiosyncrasy is important (to be discussed further); and (6) the question of the selective sensitivity of different parts of the body does not apply here.

[4] *It has been shown, in fact, in* Drosophila *that they are additive in succeeding generations.*

Dose-effect curves are not available for the other types of injury listed so that it is not possible to classify them as to single- or multiple-event types of reactions. However, since X-ray dermatitis, sterility (as distinguished from radiogenetic injury), lymphopenia, leukopenia, anemia, bone necrosis, glandular disfunction, and fetal injury fail to appear if the dosage is kept small, these reactions appear to be of the multiple-event type. While the induction of tumors or leukemia by X-rays may also be of this type, a satisfactory answer cannot be had until we know more about tumor etiology (e. g., whether X-ray tumors are of single- or multiple-cell origin).

Thus, while certain aspects of some of the reactions are not adequately understood, it appears plain, nevertheless, that we have two distinct types of reactions with which to deal in protection—types, in fact, which require very different considerations.

CONCEPTS OF PROTECTION

At the present stage in the history of protection, it is clear that the idea of tolerance dose automatically implies threshold-type reactions—that is, that there must exist, so far as the injurious effects are concerned, doses below which no effect is produced. But, as already mentioned, there exists at least one type of injury—and an important one—to which the tolerance-dose idea simply cannot be applied in the same sense. In the case of radiogenetic injury it is now apparent that for any given increment of dosage, no matter how large or how small, there exist certain chances of mutation and that these chances vary directly with the dose. Hence, instead of tolerance dose, it would seem more appropriate to speak of tolerance injury. Such an expression would imply, and correctly so, that any value that might be associated with the term would be entirely arbitrary. In such a case we would set the amount of genetic injury we are willing to tolerate and regulate accordingly the amount of radiation that will reach the gonads before childbearing.

After the dual character of the protection problem has thus been recognized, the question arises, very naturally, as to what safety value 0.1 r/day has in the case of the threshold-type reactions and the injury value it may have in the case of the non-threshold type. The published information in regard to the first is, so far as we know, all given in the table showing recommended tolerance intensities. As mentioned, these findings are based on the most limited investigations, but in view of the fact that the tolerance standard of 0.1 to 0.2 r/day has been used by a number of institutions and generally satisfactory results have been obtained, it is obviously better established than is indicated by the findings in the table so far as the more conspicuous injuries are concerned. Since only recently have we begun to be aware of the fact that radiation may be a factor in inducing leukemia, that a very long interval may occur between exposure and the appearance of radiation-induced tumor, and that such tumors may be induced without the previous appearance of precancerous lesions, these experiences furnish no adequate proof that 0.1 to 0.2 r/day is safe for all threshold reactions. Cowie and Scheele (*5*) and Uhlmann (*65*), however, indicate that irradiation injury is by no means uncommon. One gains the impression, nevertheless, from the report by Cowie and Scheele that the present skin injuries are resulting more from failure to maintain the standard rather than from an incorrect standard.

As to the safety value of 0.1 r/day in

the case of genetic injury in humans, there is no information; no curve is available as in the case of *Drosophila*, showing how the number of mutations produced varies with the dose of radiation reaching the germ cells. However, should it become advisable, as Muller has suggested, to relate the rate of induced mutations to that of the spontaneous type, it would be necessary to know something of the spontaneous rate and how constant it may be. Fortunately, there is some information on this point for lower forms.

THE CONSTANCY OF SPONTANEOUS MUTATION

Muller (*66*) and Timofeeff-Ressovsky (*60*) show, in their work on *Drosophila*, that the rate of any given kind of mutation is quite constant. For a particular lethal type, Muller has shown that it occurs (spontaneously) in about 1 out of every 1,000 germ cells. It has been found, however, that the spontaneous rate is not the same for different types of mutations. The specific organization along the body of the chromosome seems to cause the stability at various points to differ, thus causing certain types of mutations to occur more frequently than others. In addition, there are the so-called mutable genes, such as those that have to do with size, color variety, etc., which undergo changes more frequently.

From the foregoing, it would seem that while the different kinds of mutations are known to occur at different rates, one should be able to relate the rate of induced mutations of a given type to the rate of spontaneous mutations of the same type with a fair degree of reliability.

To illustrate just how such a relation might be used, let us assume that the development of feeblemindedness was known to occur spontaneously in 1 out of every 1,000 offspring, and that the normal rate was doubled by exposure to 35 r (as Muller has found for a particular effect in *Drosophila*): then if the tolerance intensity of 0.1 r/day is being maintained, the incidence of mental weakness would be increased from 1 in 1,000 to 2 in 1,000 with about 1 year of exposure. Among other things, this illustration goes to show how much information is needed, since so very little is known of mutations in humans. Lest one should gain the impression, however, that mutations are of rare occurrence, attention should be called to the fact that when all possible mutations are taken into account in *Drosophila*, lethal, sublethal, large, and small, as high as 75 percent of the chromosomes have been reported to have mutant genes (Dobzhansky and Queal (*67*) 1938).

GENERAL CONSIDERATIONS

From what has just been said, it appears that spontaneous mutation may be of more frequent occurrence than is usually suspected, and the question arises very naturally as to what extent spontaneous mutation contributes to the course of evolution. Without entering into a discussion of the subject, it seems safe to say at this stage of our knowledge that spontaneous mutation contributes some, if not a great deal, to the process of evolution. Thus, since radiation enhances the mutation rate, since the germ cells of children give rise by direct cell lineage to the germ cells that become functional in later life, and since the exposure (in adults and youth alike) is becoming constantly greater through radiography, therapy, industry, and merchandising (fitting of shoes, etc.), it seems clear that because of the use of radiation, evolution is being moved forward at an ever-increasing rate—a situation which indeed may have far-reaching social implications.

As it becomes apparent that a significant amount of mutation occurs spontaneously among cells and that radiation adds to this effect, the question arises as to whether prospects for the future use of radiation really furnish cause for alarm. If we accept the normal rate of mutation or the normal rate of evolution without concern, why then should we not be equally unconcerned if the tempo is merely stepped up one, two, or even several fold? In nature the process of natural selection through survival of the fittest is constantly in effect, thus retaining only those changes which aid in racial survival. Hence, may we not say that the same forces will be in effect if the tempo of evolution is stepped up and that the end result will be very much the same as at present? While an adequate answer is not available on this point, the outlook is against such an assumption, three reasons for which may be given: (1) Increasing the tempo of evolution would cause a greater proportion of the less fit in any given generation or at any time. This is true because time aids in a more rigorous selection of the fittest. Surely there would be universal agreement that this is undesirable. Whether the increase in less fit will be enough to cause concern depends entirely on how much the tempo is increased. (2) It appears that whereas normal mutation tends more to be associated with less stable chromosome areas where less consequential variation occurs, X-ray-induced mutation appears to be more widespread in character and more devastating, thus causing a greater proportion of harmful and lethal mutations. Lethal changes, of course, would have little or no effect on racial well-being, but they would be a matter of much concern in the first generations. By this it is meant that X-rays produce a greater proportion of changes in germ cells that cause abnormalities in development and result in abortion or infant mortality. (3) With the tendency for detrimental hereditary changes to be genetic recessives, the more rapid production of these in the general population would have the effect of replacing strong characters with weaker ones, thus leading toward racial weakness.

There is a strong tendency at this time, especially by those who are faced with the need for using X-rays to aid patients and so to save lives, to say that, since there is almost no evidence that genetic abnormalities are induced in humans, the good that they are doing far outweighs the possible damage done to future offspring. This challenge cannot be met at this time, but the indications cited above (especially by Zappert (*22*), and by Hickey and Hall (*23*)), limited as they may be, furnish ample evidence that the germ plasm of man is not excluded from the possibility of irradiation injury. The opponents of radiogenetic change may point to apparently normal sons and daughters of radiologists who have received a great deal of radiation before these children were conceived, or to menstruating daughters whose mothers received considerable pelvic preconception irradiation. These are important examples and they carry weight, but they do not prove that the unfortunate cases derived their abnormalities from sources other than radiation. It is likewise possible to point out cases of serious abnormalities in offspring of persons who received preconception irradiation, but since we are unable as yet to distinguish preconception irradiation injury from the spontaneously occurring abnormalities, such cases cannot be said with certainty to be due to the influence of irradiation. It is thus apparent that much more information is needed, particularly of the type obtained by Hickey and Hall.

While these considerations are specu-

lative and do little more than stimulate our imagination, they, nevertheless, suggest that radiation, as it will be used in the future, will in all probability exert an appreciable influence on the course of evolution. Until we know more about the nature of the process and of the desirability of the influence it will have, we are faced with the problem of what to do in the meantime. Should we curtail the exposure to radiation before the childbearing period or should we disregard the indications and trust that no unfortunate circumstances will develop? Until more information is available, surely it is preferable to take the attitude that exposure of the germ line should be avoided as much as possible. As Muller (*59*) said:

> We must remember that the thread of germ plasm which now exists must suffice to furnish the seeds of the human race even for the most remote future. We are the present custodians of this all important material and it is up to us to guard it carefully and not contaminate it for the sake of any ephemeral benefits to our own generation.

From the discussion given, it is clear that no significant indication can be made of the injury value of 0.1 r/day in the case of radiogenetic changes in humans. However, since it may have a derogatory effect, the extent of which is as yet unseen, and since the exposure of personnel is usually much lower than 0.1 r/day if the recommended physical standards of protection are followed (*5*), surely it is advisable to keep the exposure of germ cells well below the present standard of safety and to avoid exposure completely before childbearing.

PROTECTION ATTAINMENTS AND NEEDS

By way of retrospect some of the apparent protection accomplishments and some of the apparent needs for the future are listed here:

(1) A tolerance dose (usually 0.1 to 0.2 r/day) has been recommended by various protection committees. Judging more from unpublished results than from published, it would seem that so far as certain types of injury are concerned (skin damage, blood changes, general sterility), this standard provides fairly adequate protection, when general body exposure is concerned. So far as other injuries are concerned (induction of tumors, including leukemia), our experience is as yet too limited even to allow surmises. We know little of the rate of accumulation of irradiation effect in these cases, or indeed whether such is necessary, nor do we know actually whether X-ray dermatitis is a necessary forerunner of X-ray tumors.

(2) In regard to local exposure it has been emphasized by Cowie and Scheele (*5*) and by Quimby (*48*) that severe injury may be produced on the hands (particularly in the case of gamma rays) while the exposure of the body is within the limits of the safety standard. Thus, it does not seem advisable to presume, as some have done, that "since the tolerance standard contains a generous factor of safety, we need not distinguish between local and general effects." Instead, it would seem safer and more consistent to have the standard apply locally the same as generally.

(3) Heretofore, the safety standard has been made to apply almost exclusively to changes in the superficial tissues and derangements of the internal organs including changes in the blood. In view of that which has been said, it is desirable to have the safety standard defined more specifically. It is suggested, therefore, that the tolerance dose for threshold-type reactions should be related to the most sensitive injury effect known, and then that it be assumed that all other types of injury are automatically cared for. It is suggested

further that the tolerance dose in the case of whole body exposure be defined with respect to lymphopenia for two reasons: (1) Lymphocytes appear to be the most sensitive cells in the body; and (2) because a lymphocyte record provides a fairly satisfactory quantitative measure of the degree of injury when present. Since a complete blood record can be obtained almost as simply as a lymphocyte count alone, and because blood changes other than that of the lymphocyte count will give additional information, it would seem proper to define the tolerance dose for whole body exposure simply with respect to blood changes. In the case of local injury, such as on the hands, blood changes would probably have little meaning. Hence, it is necessary to use a different effect here, and it is suggested that sustained faint skin reddening be used, such as that which appears at the base of the nails of radium workers, since this is one of the first signs of damage and fairly easy to detect.

Thus following the pattern used by Taylor, we suggest that "tolerance dose" be defined as the amount of X-ray or gamma ray energy that a person can receive continuously or at repeated intervals without suffering changes in the blood when the whole body is exposed, or reddening of the skin when local areas are exposed.

SUMMARY

1. The history of protection from injury caused by X-rays and gamma rays and of the tolerance dose and the trends in protection have been discussed.

2. As a consequence, it becomes clear that practically all of the discussions on protection have pertained to the physical aspects of the problem with the result that the nature of the injuries to be protected against and the significance of the biological factors involved have been almost completely overlooked.

3. By taking account of the information now available, it is possible to point out that the previous concepts of protection do not apply to one kind of effect, the radiogenetic.

4. The term "tolerance dose" as used before can apply only to threshold-type reactions—ones for which safe exposures exist. The radiogenetic type of injury, as manifested in lower forms, is shown to have no threshold of safety. The problem, therefore, is one involving probability of action or injury.

5. Thus the question of whether the genetic injury produced by 0.1 r/day is tolerable is obviously an arbitrary matter. Unfortunately, not enough data are available for human beings to allow any satisfactory statement as to how much genetic damage is produced by this exposure; but by analogy from the condition in lower forms, this appears to be more than we should prefer to tolerate.

6. Because of the non-threshold character of the radiogenetic reaction and because of the far-reaching influence that it may have, the significance of this irradiation effect has been stressed.

7. The tolerance dose (for threshold-type reactions) is more specifically defined as the amount of X-ray or gamma ray energy that a person can receive continuously or at repeated intervals without suffering changes in the blood when the whole body is exposed or sustained redness of the skin when local areas are concerned, the assumption being that if these effects are protected against, all other types of injury will be cared for automatically.

8. Much more information is needed before the adequacy of 0.1 r/day as a safety standard can be stated. Accordingly, available information pertaining

to persons (especially groups of persons) who have been exposed to known or reasonably well estimated amounts of radiation for known periods of time, and whose health records contain information about blood and skin changes, reproductive potencies, etc., should be published at the earliest opportunity. Likewise, records should be kept where possible in order that future tabulations can be made.

REFERENCES

(*1*) INTERNATIONAL X-RAY AND RADIUM COMMISSION: International recommendations for X-ray and radium protection. Radiology, 23: 682–685 (1934).

(*2*) ———: International recommendations for X-ray and radium protection. Radiology, 30: 511–515 (1937).

(*3*) ADVISORY COMMITTEE ON X-RAY AND RADIUM PROTECTION: X-ray protection. Bur. Standards Handbook 15 (1931).

(*4*) ———: X-ray protection. (Revised). Nat. Bur. Standards Handbook 20 (1936).

(*5*) COWIE, D. B., and SCHEELE, L. A.: A survey of radiation protection in hospitals. J. Nat. Cancer Inst., 1: 767–787 (1941).

(*6*) MARCUSE, W.: Dermatitis und Alopecie nach Durchleuchtungsversuchen mit Röntgenstrahlen. Deutsche med. Wchnschr., 22: 481–483 (1896).

(*7*) TAYLOR, L. S.: Roentgen-ray protection. *In* Glasser, O., ed., The Science of Radiology, pp. 332–343. Charles C. Thomas, Baltimore (1933).

(*8*) LEDDY, E. T.: Protection in X-ray and radium therapy. *In* Pack, G. T., and Livingston, E. M., Treatment of Cancer and Allied Diseases . . . Paul B. Hoeber, Inc., New York (1940).

(*9*) FRIEBEN: Demonstr. Ärztl. Verein, Hamburg, Oct. 21, 1902. Cited by Colwell and Russ (*16*).

(*10*) HESSE, O.: Das Röntgenkarzinom. Fortschr. a. d. Geb. d. Röntgenstrahlen, 17: 82–92 (1911).

(*11*) MILCHNER, R., and MOSSE, M.: Zur Frage der Behandlung der Blutkrankheiten mit Röntgenstrahlen. Berlin. klin. Wchnschr., 41: 1267–1269 (1904).

(*12*) HEINEKE, H.: Ueber die Einwirkung der Röntgenstrahlen auf innere Organe. München. med. Wchnschr., 51: 785–786 (1904–5).

(*13*) ———: Experimentelle Untersuchungen über die Einwirkung der Röntgenstrahlen auf das Knochenmark nebst einigen Bemerkungen über die Röntgentherapie der Leukämie und Pseudoleukämie und des Sarkoms. Deutsche Ztschr. f. Chir., 78: 196–230 (1905).

(*14*) ALBERS-SCHÖNBERG, H.: Ueber eine bisher unbekannte Wirkung der Röntgenstrahlen auf den Organismus der Tiere. München. med. Wchnschr., 50: 1859 (1903).

(*15*) BERGONIÉ, J., and TRIBONDEAU, L. P.: Action des rayons X sur le testicule du rat blanc. Compt. rend. Soc. de biol., 57: 595 (1904).

(*16*) COLWELL, H. A., and RUSS, S.: X-ray and radium injuries: Prevention and treatment. Oxford M. Pubs., New York and London (1934).

(*17*) WALSH, D.: Focus tube dermatitis. Arch. Roentgen Ray, London, 3: 69–74 (1898–99).

(*18*) PERTHES, G.: Röntgenschaden, 1903. Cited by Colwell and Russ (*16*).

(*19*) MARTLAND, H. S.: The occurrence of malignancy in radioactive persons . . . Am. J. Cancer, 15: 2435–2516 (1931).

(*20*) HAAGENSEN, C. D.: Occupational neoplastic disease. Am. J. Cancer, 15: 641–703 (1931).

(*21*) READ, J., and MOTTRAM, J. C.: The "tolerance concentration" of radon in the atmosphere. Brit. J. Radiol., 12: 54–60 (1938).

(*22*) ZAPPERT, J.: Hat eine Strahlenbehandlung der graviden Mutter einen schädlichen Einfluss auf das Kind? Wien. klin. Wchnschr., 38: 669–673 (1925).

(*23*) HICKEY, P. M., and HALL, E. W.: A report analyzing the results of the questionnaire sent out to radiologists, under the direction of the Sex Committee of the National Research Council. Am. J. Roentgenol., 18: 458–462 (1927).

(*24*) MURPHY, D. P.: Outcome of 625 pregnancies in women subjected to pelvic radium or roentgen irradiation. Am. J. Obst. & Gynec., 18: 179–187 (1929).

(*25*) ———: Maternal pelvic irradiation as the cause of microcephaly. J. Psycho-Asthenics, 34: (1929).

(*26*) ABELS, H.: Ueber Hemmungsbildungen an einem Neugeboren durch Röntgeneinwirkung in früher Fötalperiode. Wien. klin. Wchnschr., 37: 869–870 (1924).

(*27*) ROLLINS, W.: Vacuum tube burns. Boston Med. & Surg., 146: 39 (1902).

(*28*) RUSS, S.: Hard and soft X-rays. Arch. Roentgen Ray, London, 19: 323–325 (1914–15).

(*29*) BRITISH X-RAY and RADIUM PROTECTION COMMITTEE: X-ray and radium protection. J. Roentgen. Soc., 17: 100–103 (1921).

(*30*) AMERICAN ROENTGEN RAY SOCIETY: Proceedings of the annual meeting . . . (1920).

(*31*) KAYE, G. W. C.: The story of protection (Stanley Melville memorial lecture). Radiography, 6: 41–60 (1940).

(*32*) TAYLOR, L. S.: X-ray protection. J. A. M. A., 116: 136–140 (1941).

(*33*) MUTSCHELLER, A.: Physical standards of protection against roentgen-ray dangers. Am. J. Roentgenol., 13: 65–70 (1925).

(*34*) SIEVERT, R. M.: Einige Untersuchungen über Vorrichtungen zum Schutz gegen Röntgenstrahlen. Acta radiol., 4: 61–75 (1925).

(*35*) GLOCKER, R., and KAUPP, E.: Über den Strahlenschutz und die Toleranzdosis. Strahlentherapie, 20: 144–152 (1935).

(*36*) SOLOMON: Précis de radiothèrapie profonde. (1926). Cited by Kaye (*41*).

(*37*) DUTCH BOARD OF HEALTH: Fundamental aspects of roentgen rays. Cited by Kaye (*41*).

(*38*) BARCLAY, A. E., and COX, S.: The radiation risks of the roentgenologist; attempts to measure quantity of roentgen rays used in diagnosis and to assess dangers. Am. J. Röentgenol., 19: 551–561 (1928).

(*39*) BOUWERS, A., and VAN DER TUUK, J. H.: Strahlenschutz. Fortschr. a. d. Geb. der Röntgenstrahlen, 41: 767–776 (1930).

(*40*) FAILLA, G.: Radium protection. Radiology, 19: 112–21 (1932).

(*41*) KAYE, G. W. C.: Roentgenology: Its early history, some basic principles and protective measures. Paul B. Hoeber, Inc., New York (1928).

(*42*) (UNITED STATES) BUREAU OF STANDARDS: X-ray and radium protection. Bur. Standards Circular No. 374 (1929).

(*43*) BRITISH INSTITUTE OF RADIOLOGY: International recommendations for X-ray and radium protection. *In* British Institute of Radiology Year Book (1932).

(*44*) TAYLOR, L. S.: The work of the national and international committees on X-ray and radium protection. Radiology, 19: 1–4 (1932).

(*45*) BRAESTRUP, C. B.: A stray radiation survey of twenty high voltage installations. Radiology, 31: 206–213 (1938).

(*46*) EVANS, R. D.: Radium poisoning: A review of present knowledge. Am. J. Pub. Health, 23: 1017–1023 (1933).

(*47*) STENSTROM, W.: Protection in X-ray therapy. Radiology, 19:7–11 (1932).

(*48*) QUIMBY, E. H.: Radium protection. J. Appl. Phys., 10: 604–608 (1939).

(*49*) KAYE, G. W. C., BELL, G. E., and BRINKS, W.: The protection of radium workers from gamma radiation. Brit. J. Radiol., 8: 6–26 (1935).

(*50*) MULLER, H. J.: Artificial transmutation of the gene. Science, 66: 84–87 (1927).

(*51*) MURPHY, D. P.: Ovarian irradiation; its effect on the health of subsequent children . . . Surg., Gynec. & Obst., 47: 201–215 (1928).

(*52*) MURPHY, D. P.: Ovarian irradiation and the health of the subsequent child; a review of more than 200 previously unreported pregnancies in women subject to pelvic irradiation. Surg., Gynec. & Obst., 49: 766–779 (1929).

(*53*) MURPHY, D. P., and GOLDSTEIN, L.: Etiology of the ill-health of children born after maternal pelvic irradiation. I. Unhealthy children born after preception pelvic irradiation. Am. J. Roentgenol., 22: 207–219 (1929).

(*54*) MURPHY, D. P.: Preconception ovarian irradiation, its influence upon the descendants of the albino rat (Mus norvegicus). Surg., Gynec. & Obst., 50: 558–593 (1930).

(*55*) NAUJOKS, H.: Fertilität und Nachkommenschaft früherer Röntgenassistentinnen. Ein Beitrag zur Frage der sog. "Keimschädigung." Strahlentherapie, 32: 613–639 (1929).

(*56*) SNELL, G. D., and AMES, F. B.: Hereditary changes in the descendants of female mice exposed to roentgen rays. Am. J. Roentgenol., 41: 248–255 (1939).

(*57*) HERTWIG, P.: Erbänderungen bei Mausen nach Röntgenstrahlung. Proc. 7th Internat. Genetics Cong., Suppl. to J. Genetics (1939).

(*58*) SINNOTT, E. W., and DUNN, L. C.: Principles of genetics. Ed. 3. McGraw-Hill Book Co., Inc., New York and London (1939).

(*59*) MULLER, H. J.: The effects of roentgen rays upon the hereditary material. *In* Glasser, O., ed., The Science of Radiology, pp. 305–318. Charles C. Thomas, Baltimore (1933).

(*60*) TIMOFEEFF-RESSOVSKY, N. W.: Mutationsforschung in der Vererbungslehre. Theodor Steinkopf, Dresden and Leipzig (1937).

(*61*) SCHULTZ, J.: Radiation and the study of mutation in animals. *In* Duggar, B. M., ed., Biological Effects of Radiation . . . McGraw-Hill Book Co., Inc., New York and London (1936).

(*62*) SUGIURA, K.: Studies on radiosensitivity of mouse sarcoma 180 irradiated in vivo and in vitro. Radiology, 29: 352–361 (1937).

(*63*) GOODSPEED, T. H., and UBER, F. M.: Radiation and plant genetics. Bot. Rev., 5: 1–48 (1939).

(*64*) SAX, K.: An analysis of X-ray induced chromosomal aberrations in Tradescantia. Genetics, 25: 41–68 (1940).

(*65*) UHLMANN, E.: The treatment of injuries produced by roentgen rays and radioactive substances. Am. J. Roentgenol., 41: 80–90 (1939).

(*66*) MULLER, H. J.: Radiation and genetics. Am. Nat., 64: 220–251 (1930).

(*67*) DOBZHANSKY, T., and QUEAL, M. L.: Genetics of natural populations. II. Genic variation in populations of Drosophila pseudoobscura inhabiting isolated mountain ranges. Genetics, 23: 463–484 (1938).

(*68*) WESTING, W. W.: The importance of new X-ray effect for our daily diagnostic and therapeutic X-ray work. New York State J. Med., 40: 1139–1144 (1940).

(*69*) PFAHLER, G. E.: The effects of X-rays and radium on the blood and general health of radiologists. Am. J. Roentgenol., 9: 647–656 (1922).

(*70*) Protection from X-rays and radium. Lancet 225: 1060 (1933).

Quantitative Analysis of Dose-Response Data Obtained With Carcinogenic Hydrocarbons

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INTRODUCTION

During the past few years a large amount of work has been performed on the biological response to various chemicals possessing the property of inducing neoplasia (*1–4*). The wide variety of the investigations served to ascertain the most desirable species and strains of animals, technical procedures, and other conditions for the practical purposes of experimentation (*5*). It was inevitable that, with the accumulation of appropriate qualitative findings, there was an intensification of the studies toward the quantitative expression of the reactions.

Many types of agents which produce characteristic biological effects in animals have been found to act quantitatively in relation to the administered dose or concentration. A system of biomathematical procedures applicable to the quantitative analysis of dose-response data has been developed during recent years. The use of these methods has been found to be not only desirable but also indispensable in investigations with various biologically active materials, including drugs (*6–10*), vitamins (*11–12*), hormones (*13–15*), and viruses (*16–20*).

Abundant evidence available in the literature indicates that a quantitative relation exists between the dose of the chemically pure carcinogenic hydrocarbons and the neoplastic response. It is of interest to ascertain whether these apparent relations between dose and response are subject to mathematical expression and whether the existing analytical methods are applicable to the purpose. It is felt that value would accrue from the application of the biomathematical procedures to data with carcinogens, not only in the examination of the data available at present but also in the pursual of future experiments.

The primary objective of this study, therefore, is to ascertain whether the statistical methods which have been developed for the analysis of dose-response data are applicable in their present forms to results with carcinogenic hydrocarbons. For this purpose, the most suitable data from the literature are used. Since the methods are found to be applicable, they are applied to certain problems which are encountered in studies with carcinogenic agents.

For convenience, the report is divided into three main parts which deal, respectively, with (I) a discussion of the methods, (II) proof of their applicability to data obtained with carcinogens, and (III) their application to certain specific problems.

PART I: DISCUSSION OF THE METHODS

The fundamental principles of the analytical procedures are the same whether the purpose of experimentation is to study

[1] *We are indebted to Dr. Harold F. Dorn for his valuable suggestions and criticisms.*

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the animal response to known quantities of an active agent or whether it is to assay an unknown material on the basis of animal response. The principles are more readily summarized, however, from the latter standpoint.

In carrying out a bioassay, an "unknown" or test preparation of a given material is compared with a standard preparation of the same material on the basis of the respective quantities required in each case to give a common animal response. In such comparison it is essential that all variable factors other than the one being measured (e. g., potency) be controlled or allowed for in the interpretation of the results. The animal response to a given dose of a preparation may vary markedly with different experimental procedures (e. g., methods of administration) or with different populations of test animals (species, strains, age groups, etc.), but for a constant set of conditions the relation between dose and response is usually characteristic and reproducible within determinable limits of error. Any expression of dose in terms of animal response, or vice versa, is obviously relative, but under constant conditions real differences in the biological potencies of various preparations can be determined by common reference to a standard sample of the same material.

Although important sources of variation are controlled by the use of constant techniques and a constant population of test animals, there is another factor which cannot be controlled experimentally and which, therefore, must be allowed for in the interpretation of results. This important consideration is the variation among individual animals within a given population. Recognition of the true extent of the variations due to this source began in 1927 with the work of Trevan (*6*) on the toxicity of digitalis in frogs. In spite of a wide range of variation, he showed that the variations occurred in a systematic way and that a regular, as well as a characteristic relation could be evinced between dose and animal response if adequate numbers of test animals were used.

Where systematic variables of this sort must be considered in the interpretation of results, the system of mathematics which must be employed is the statistical method. After Trevan's demonstration of their needs, statistical techniques and procedures for the quantitative analysis of dose-response data were evolved, chiefly by workers in the field of pharmacology (*6–14*). The methods were made available for general use by the comprehensive reports of Gaddum (*7*), of Bliss (*8, 21, 22*), and of Irwin (*12*). Gaddum provided the mathematical basis for analyses involving the quantal type of response. Bliss introduced valuable techniques and tables which simplified the application of these principles so that analyses could be performed by those who do not have a previous knowledge of statistics. Irwin published a systematic treatment of the broader aspects of the statistical method applied to biological assays and included the measurable as well as the quantal type of biological response. A general consideration of both the technical and the analytical aspects of the subject of biological assays was presented by Burn (*9*).

With the growing use of the statistical methods in the analysis of animal responses, adaptations of the established principles and statistical techniques to specific problems have furnished numerous examples of treatment of data obtained in various types of investigation. Thus, in his studies on variables affecting the estimation of androgenic and estrogenic activity, Emmens (*14*) introduced procedures which are applicable not only to this particular problem but which may be used in other fields. Important also in this respect is

the standardization of the safety margin of drugs which have both a lethal and a therapeutic effect. The solution to this thorny and recurring problem was given by Foster (*10*). In addition to illustrating specific mathematical procedures of general usefulness, Foster also suggested the standardization of certain terms and notations along the lines first proposed by Trevan (*6*). The importance of such standard terminology is obvious, and in subsequent discussions the terminology given by Foster is followed wherever applicable.

In its relation to the quantitative analysis of dose-response data, the statistical method serves two chief purposes: (1) It allows estimation of the variability among individual test animals and hence estimation of the reliability of the average or group response to a given dose; and (2) it permits estimation of the quantitative relation between group response and the administered dose, i. e., the trend of group responses over a wide range of doses. If it were possible to estimate average or group responses within negligible limits of error, the relation between dose and response could also be determined without error, and statistical treatment of the data would be unnecessary. Results obtained with various preparations of a given material could then be compared with results obtained on a standard preparation by the use of simple algebraic or graphic procedures. With practical numbers of test animals, however, it is usually found that estimates of average or group responses are subject to appreciable error which must be allowed for in the interpretation of results. Furthermore, the group responses observed at various dose levels will not lie exactly on the smooth curve which represents the best estimate of the trend of the data. By the use of statistical methods it is possible to establish limits of deviation within which average or group responses will lie as long as experimental conditions are unchanged.

It is desirable to have a linear relationship between dose and response since it is easier to fit a straight line to variable data of this sort than to fit any other type of curve. Furthermore, the statistical analysis of the data is greatly simplified when the relation is linear. Experience has shown that in most cases suitable units can be found for converting dose-response data to a linear relationship, and the statistical techniques which have been developed for the analysis of such data are most readily applied when a linear transformation is possible. The test of whether the established statistical techniques are applicable in any particular case, then, is a test for a linear relationship between dose and average or group response. If this condition can be satisfied, a wealth of well-grounded procedures is available for the statistical analysis of the data. If the condition cannot be satisfied, special methods have to be employed (cf. Irwin (*12*)).

Types of Response

The present study is concerned only with the local response, i. e., development of tumors, at the sites of subcutaneous injection of carcinogenic polycyclic aromatic hydrocarbons. The induction of tumors in loci remote from the sites of injection, e. g., in the lungs (*23*), will be considered in a subsequent publication.

The development of tumors at the site of injection involves two types of response which vary with the magnitude of the dose administered, namely, (1) the latent period and (2) the frequency of positive injection sites or the incidence of tumors.

Latent Period

As the term is used in its present sense, latent period represents the period of time

elapsing between the injection of a given dose of a carcinogenic agent and the appearance of a tumor. The criterion for determining the time of appearance is simply the discovery, on palpation, of a discrete, progressively growing nodule at the site of injection. For example, a palpable nodule which proves on subsequent examination at appropriate intervals to be a progressively growing mass and by an ultimate histological examination to be a malignant neoplasm, is recorded as having appeared on the date on which it was first discovered.[2] The accuracy of such estimates is obviously influenced both by the rate of tumor growth and by the interval of time between routine examinations for the presence of tumors. Regardless of refinements in the technique of observation there are certain limits of error associated with this sort of observations which cannot be further reduced. Consequently, in most cases the latent period for a given tumor can be established only within limits of error of several days to a week.

The latent-period response is a variable of the type referred to by the statistician as a *continuous variate* (*12*). That is, the response of each individual test animal can be measured quantitatively, and the group response (where all members of the group have received exactly the same dose) can be expressed as an average of the individual responses. When the biological response can be thus measured, it is generally found that the average responses of large groups of animals are linearly related to the logarithm of dose and not to dose on an arithmetic scale.

The conversion of dosages to logarithmic values is, therefore, the only transformation of the data usually required to give the desired linear relationship between dose and response. In subsequent sections it is shown that such is the case with dose-latent period data obtained with carcinogenic hydrocarbons.

The analysis of data when the response is a continuous variate and when the relation between average response and log dose is linear is discussed in detail by Irwin (*12*).

Tumor Incidence

The incidence of tumors in a group of animals, all members of which have received exactly the same dose of a given carcinogenic agent, is known to vary with the magnitude of the dose administered. The percentage of animals which show some characteristic biological effect is a type of response which has been widely used in quantitative studies on numerous biologically active materials. Responses of this sort are designated as quantal, or "all-or-none" (*7*, *12*), since all treated animals fall into either one or the other of two opposite categories, namely, that of animals which show the characteristic effect, and that of animals which fail to show the effect. Insofar as the individual animal is concerned, the experimental observation is simply a qualitative determination of the category into which it falls.

When the individual response is quantal, the group response is expressed as a proportion or percent. For example, the number of animals showing the characteristic effect is expressed as a proportion, or percent, of the total number which could have shown the effect, namely, the total number in the test group.

In a typical experiment in which increasing doses are administered to successive groups of animals there is found a zone of doses in which no animal shows the characteristic effect, a zone in which all ani-

[2] *Recent data* (24) *indicate that the death of mice with subcutaneous sarcomas is a reliable criterion for the latent period, and that the mean difference between the period at which tumors become palpable and the death of the animals is constant.*

mals show the effect, and an intermediate zone in which the percent showing the effect increases as the dose is increased. The response curve obtained when percent is plotted against dose on a logarithmic scale is S-shaped in the region corresponding to the intermediate zone. Above and below certain dose limits the curve approaches 100 percent and 0 percent, respectively, as asymptotes. The S-shaped curve is usually characteristic for any given set of constant conditions; and when constant technical procedures are employed, it may be considered as representing primarily the uncontrollable variations due to differences among the responses of individual test animals (*7*, *8*, *12*). Such factors as variations in technical procedure and errors in the quantities of the material administered also influence the results, but under carefully controlled conditions the variations from these sources are usually negligible as compared with animal variations. The S-shaped curve may therefore be considered for all practical purposes as representing a distribution of the variations due to biological factors. In the majority of cases where it has been possible to determine the exact quantities of a given material required to produce a quantal response in various individuals of a given population it has been found that the logarithms of the "individual effective doses" are normally distributed (*12*). With many materials, however, it is impossible to determine experimentally the exact quantity required to produce an effect in a single animal. Instead, a large group of animals must be treated with a given dose in order to determine the percentage of the group which shows the characteristic response. This estimates the percentage of the group which has "individual effective doses" equal to, or less than the dose administered. When frequencies of individual responses of the former type are plotted against the logarithm of dose, normal distribution curves are obtained. As pointed out above, however, observations of the latter sort yield S-shaped curves when plotted against the logarithm of dose. It has been demonstrated (*9*) that both types of curves represent fundamentally the same variable factors, namely, the variations in individual test animal susceptibilities as represented by the quantities of material required to just cause an effect in the various individuals. The S-shaped curve may therefore be considered as an integrated normal frequency curve representing the accumulated percentage frequencies of individual effective doses. As such it may be used to estimate the median individual effective dose for the particular animal population involved since the 50-percent point on the S-shaped curve corresponds to the median, as well as to the mean, of the normal curve. The standard deviation of individual effective doses may also be estimated from the S-shaped curve. The method for obtaining this estimate has been described by Gaddum (*7*).

Since the response curve may be considered as an integrated normal curve, advantage may be taken of this fact to convert the S-shaped curve into a linear form. Gaddum (*7*) has given the theory and method for the transformation which may be stated briefly as follows: Percentage values on an S-shaped dose-response curve may be considered as percentages of the total area of a normal curve. From the fixed relation which exists between a deviate marked off in standard deviation units along the abscissa of a normal curve and the proportionate area of the curve which would be cut off by a vertical line drawn at this deviate, it is possible to express proportionate areas (or percent) in terms of deviate, and vice versa.

Gaddum (*7*) called the deviate, in standard deviation units, corresponding to a given percent value a *normal equivalent deviation*. Since normal equivalent deviations represent points along the abscissa of the normal curve, as do also the logarithmic values of the doses employed in the experiment, it follows that there is a linear relation between normal equivalent deviation and the logarithm of dose.

Published tables are available for finding the normal equivalent deviation (i. e., deviate in standard deviation units) corresponding to any percentage value (i. e., proportionate area of a normal curve) (*25, table 1*). By reference to such tables percentage values may be readily converted to normal equivalent deviations and plotted against the logarithm of dosage. When this is done, a straight-line relationship is obtained in instances in which the logarithms of individual effective doses are normally distributed.

In actual practice, the values for normal equivalent deviations fall well within the limits -5 and $+5$. The mean of the normal curve is at zero deviate, and the normal equivalent deviation of 50 percent is, therefore, 0. Accordingly, percentage values below 50 will have negative normal equivalent deviations while those above 50 percent will correspond to positive values. In order to avoid negative quantities in calculations, Bliss (*8*) added $+5$ to all normal-equivalent-deviation values and designated the new units thus obtained as *probits*. Thus the range of probit values is between 0 and 10, while the probit corresponding to 50 percent is 5. Tables for the direct conversion of percentage values to probits have been published by Bliss (*8*) and are included by Fisher and Yates (*26*) in their accumulated tables. Additional tables which aid in the calculations involving probits have also been published by Bliss (*8*).

The analysis of data when the response is quantal or "all-or-none" is described in detail by Gaddum (*7*), Bliss (*8, 21, 22*), and Irwin (*12*).

PART II: APPLICABILITY OF THE PROCEDURES TO PRESENT DATA

In the preceding section it was pointed out that methods are available for the quantitative analysis of dose-response data when the relation between response and the logarithm of dose is linear.[3] The test for the applicability of the established methods to the analysis of data obtained with carcinogenic hydrocarbons is therefore a test for the goodness of fit to a straight line of the observed relations between response and log dose. In the present section the data of several investigators whose results are given in sufficient detail for the purpose are tested in this respect.

Applicability to Tumor-Incidence Data

The following analyses were undertaken to ascertain whether there is a linear relation between response expressed in terms of probits (i. e., percentage response transformed to special probability units) and the logarithm of dose.

Data of Dobrovolskaia-Zavadskaia

Dobrovolskaia-Zavadskaia (*27*) tried to find the minimal dose of 1, 2, 5, 6-dibenzanthracene [4] which would produce tumors in mice. She gave single subcutaneous injections of from 0.01 to 0.00125 mg. of the hydrocarbon dissolved in 1/40 or 1/80 cc. of olive oil to a large number of male and female mice of unstated genetic constitution. Although the dose range represents a small fraction of the total

[3] *When the relationship is not linear it is necessary to modify the procedure to take account of nonlinearity. This requires a statistical study of the data for selection of the suitable procedures.*

[4] *Hereinafter referred to as dibenzanthracene.*

TABLE 1.—*Summary of data of Dobrovolskaia-Zavadskaia* (27) *showing percent response at various dose levels*

[Dibenzanthracene in mice]

Dose in milligrams	Mice injected	Mice with tumors	Tumor incidence	"Weighting coefficient" [1]	Dose in log milligrams (x)	Tumor incidence in probits (y)	"Weight" [2] (w)
	Number	*Number*	*Percent*				
0.01	328	37	11.28	0.366	−2.000	3.788	120.05
0.005	364	13	3.57	.180	−2.301	3.197	65.52
0.0025	167	2	1.20	.083	−2.602	2.742	13.86
0.00125	158	0	0	.010	−2.903	[3] 1.877	1.58

[1] Values corresponding to observed responses, percent or probits, as determined from Bliss' table III (*8*).
[2] Obtained by multiplying the number of mice injected (column 2) by the weighting coefficient (column 5).
[3] Estimated probit for this zero percent response as determined from trend of other data by use of Bliss' table II.

significant range, the large number of animals, as given in table 1, makes the study particularly desirable for testing the applicability of the statistical techniques to this type of material. The inadequate presentation of the survival of the animals necessitates the calculation of the percent responses from the number of tumors in the total number of mice in each particular group as given in her report.

The data are summarized in the first four columns of table 1. As pointed out in part I, it is necessary in order to obtain a linear relation between dose and percentage response to convert dosage values to logarithms and percentage values to probits. The transformed values are given in table 1, columns 6 and 7, respectively. The probit values of column 7 were obtained from the percentage values of column 4 by the use of Bliss' table I (*8*, *26*) in much the same way as the logarithms of column 6 were obtained from the values in column 1 by the use of common log tables.

In order to calculate the dose-response curve, it is necessary to know the "weight", or relative significance, of each observation. The variance of the percent response is not the same at all levels, and the weight will be influenced by the magnitude of the probit (or percentage) value as well as by the number of experimental animals. A table for estimating the weight factor associated with percentage or probit values, independent of the number of animals, has been published by Bliss (*8*, *table III*), and by use of this table, the "weighting coefficients" of column 5 were obtained.

Following the procedures described by Bliss (*8*), the equation to the regression line calculated from data in table 1 was found to be

$$Y=3.508+1.829\ (x+2.147)$$

where Y represents the calculated value of the probit corresponding to an assigned value of x, or dose in log milligrams. That the observed probit values fall within limits of random sampling about this calculated regression line is shown by the results of the χ^2 test in which $\chi^2=0.934$ and $P=0.63$.

Although the doses used in this experiment do not cover a very wide logarithmic range, the unusually large number of animals employed for each dose gives increased significance to the observations which indicate that the response curve is linear, at least in this range of doses.

Data of Lettinga

Lettinga (*28*) has published a study which involved nine doses of dibenzanthracene in mice. The procedure of administration differed from that of Dobrovolskaia-Zavadskaia in that the total dose was divided into five fortnightly injections. The hydrocarbon was dissolved in filtered lard, and each mouse received 0.5 cc. of the solution at each injection. The author

TABLE 2.—*Summary of data of Lettinga* (28) *showing percentage response at various dose levels*

[Dibenzanthracene in mice]

Dose in milligrams	Injected mice		Mice with tumors	Tumor incidence	"Weighting coefficient"	Dose in log milligrams (x)	Tumor incidence in probits (y)	"Weight" [1] (w)
	Original total	Corrected total						
	Number	*Number*	*Number*	*Percent*				
5.0	10	7.22	5	69.25	0.581	0.699	5.503	4.19
2.5	10	8.00	8	100.0	.010	.398	[2] 8.123	.08
1.0	10	9.18	9	98.0	.110	.000	7.062	1.01
0.5	10	10.00	10	100.0	.130	−.301	[2] 7.019	1.30
0.25	30	28.83	26	90.2	.336	−.602	6.295	9.69
0.125	20	16.73	9	53.8	.630	−.903	5.095	10.54
0.05	10	8.83	4	45.3	.630	−1.301	4.882	5.56
0.0125	20	12.40	4	32.3	.580	−1.903	4.539	7.19
0.005	20	7.80	0	0	.150	−2.301	[2] 3.061	1.17

[1] Obtained by multiplying the corrected total (column 3) by the weighting coefficient (column 6).
[2] Estimated probit values for zero, or 100 percent, responses determined from trend of the other observed points by use of Bliss' table II (*8*).

presented descriptions of each individual animal but did not indicate the genetic constitution of his stock.

The data are subsequently compared with those of Dobrovolskaia-Zavadskaia (*27*) despite these differences in experimental technique, since they constitute the only other complete report in the literature with doses of dibenzanthracene in mice covering a sufficiently wide range. Moreover, it has been indicated that at least at 0.25-mg. doses of this hydrocarbon, the division of the total dose into several periodic injections does not significantly alter the tumor-induction response as compared with a single administration, and that the amount of solvent, within certain limits, does not alter the reaction (*5*).

The percent responses in Lettinga's groups of animals are calculated from "corrected totals,"[5] as derived from the primary data of the original paper.

Table 2 summarizes Lettinga's data and gives the probits determined as described hitherto. The numbers of mice in the various groups are small (10 to 30) in comparison with those of the preceding experiment (158 to 364). The observed results, therefore, are much more variable.

Examination of the results in table 2 shows that the highest dose, 5.0 mg., gave a lower percent response, 69 percent, than would be expected from the trend of the remainder of the data, particularly since the preceding three successive twofold increases in dose yielded 100-percent responses. The low response with 5.0 mg. suggests that the conditions were altered and that the response to this high dose does not follow the same trend as do the responses to the smaller doses. In fact, the practical limit to the range in which percent response is correlated with dose was reached at about 1.0 to 2.0 mg. Other evidence (*5*) indicates that spurious percentage results may be obtained at high doses because of excessive tissue damage and general toxicity.

The discordant result at 5.0 mg. was therefore omitted in the analysis. It was then considered with the trend of the other data to see whether it fell within the limits of random sampling. The probability that this discrepancy was due to chance variation alone was very slight (P=0.032), and it is probable that other factors complicated the response at this dose level. Considering the upper limit of the dose

[5] *The problem of the determination of percent response, involving the correction for extraneous mortality, is considered fully under pt. III.*

range within which the response is highly correlated with dose (i. e., the range within which the response progresses from 0 to 100 percent) to be at about 1.0 to 2.0 mg., the regression line was calculated for the data falling within this significant range. The equation to the regression line was found to be

$$Y=5.366+1.439\ (x+1.070)$$

where x and Y have the same significance as described in the preceding section. Again the fit of observed responses, in probits, to the best straight line drawn through them is good ($\chi^2=5.677$, and $P=0.46$), and the relation between probit and log dose may be considered as linear.

Comparison of Results of the Two Studies

Preliminary plotting of the results obtained in the independent studies (*27*, *28*) referred to above indicated that the two sets of data are so nearly the same that they can be profitably combined to give a better estimate of the dose-response relations obtained with dibenzanthracene in mice. The continuity of trend obtained in the different dose ranges of the two studies is seen by inspection of the plotted points of figure 1.

Statistical tests indicate that there is no discrepancy between either the slopes or the positions of the respective calculated regression lines of the two studies. This does not imply that the conditions of the two experiments were the same, since there are known differences in the technical procedures and possible differences in the genetic constitution of the mice used by Dobrovolskaia-Zavadskaia and by Lettinga. The close identity of the two sets of results may be merely coincidental, because of cancellation of various differences, but statistically the two studies can be considered to be the same.

The results were combined in the manner described by Bliss (*8*), and a new regression line was calculated for the combined data. That the observed values of the two studies fall within limits of random sampling about the best straight line drawn through them is shown by the results of the χ^2 test in which $\chi^2=7.148$ and $P=0.711$. The equation to the regression line is

$$Y=3.789+1.669\ (x+1.981)$$

This line is shown graphically in relation to the observed values by the solid line of figure 1. The curved dash lines represent limits of error of the regression line corresponding to $P=0.05$.

From the foregoing analyses it can be concluded that the relation between response in probits and the logarithm of dose of dibenzanthracene in mice is satisfactorily described by a straight line. Corollary to this is the conclusion that the logarithms of the individual effective doses of this hydrocarbon in mice are normally distributed.

The established statistical procedures (pt. I) for the analysis of dose-response data

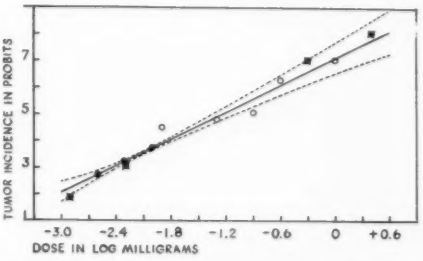

FIGURE 1.—Dose-response curve for dibenzanthracene in mice, showing regression of probits on log dose.

Solid circles: data of Dobrovolskaia-Zavadskaia (*27*).

Open circles: data of Lettinga (*28*).

Crossed circles: estimated values for 0- and 100-percent responses.

Broken lines: limits of error of response curve ($P=0.05$); these lines do not apply to the variation of the individual plotted points but to the calculated regression line.

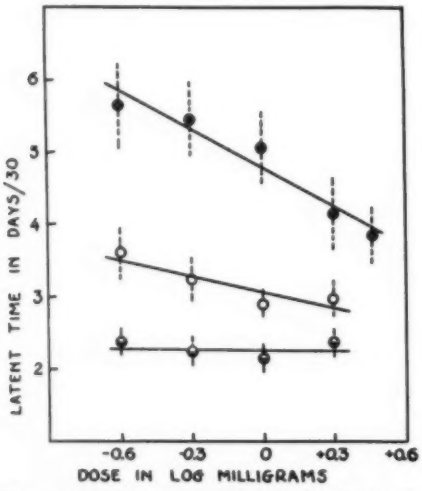

FIGURE 2.—Dose-response curves for three carcinogenic hydrocarbons in mice, showing regression of latent period on log dose. Data of Shimkin and Andervont (*29*).

Solid circles: dibenzanthracene.
Open circles: benzpyrene.
Half-solid circles: methylcholanthrene.
Vertical broken lines: limits of error for plotted means ($P=0.05$).

when the response is of the quantal type, therefore, are applicable to the analysis of results obtained with dibenzanthracene in mice.

APPLICABILITY TO LATENT-PERIOD DATA

The following analyses were undertaken to ascertain whether there is a linear relation between latent periods and the logarithm of dose of carcinogenic hydrocarbons.

Data of Shimkin and Andervont

Shimkin and Andervont (*29*) studied the response of male mice of strain C3H to 4 or 5 logarithmically spaced single doses of 20- methylcholanthrene,[6] 3, 4-benzpyrene,[7] and dibenzanthracene dissolved in 0.2 cc. of tricaprylin.

It is obvious from the summarized results of table 3, as well as from the lowest curve of figure 2, that no correlation between dose and response was obtained in the study with methylcholanthrene. An analysis of the variance shows that there is no significant variation between the latent-period responses at the various dose levels included in the study. As pointed out by the authors, the lack of correlation in this case is due to the fact that there is a minimal period of time below which tumors cannot be induced regardless of the magnitude of the dose of the carcinogen administered. Such correlation might be expected, however, in further studies with smaller doses.

The latent periods obtained with benzpyrene are shown by the summarized data of table 3 and by the group means

[6] *Hereinafter referred to as methylcholanthrene.*
[7] *Hereinafter referred to as benzpyrene.*

TABLE 3.—*Summary of latent-period data of Shimkin and Andervont* (29) *showing mean latent periods and standard deviations obtained at various dose levels*

[Data for 3 hydrocarbons in mice]

Dose in milligrams	Dose in log milligram	Methylcholanthrene			Benzpyrene			Dibenzanthracene		
		Mice with tumors	Mean latent period in days/30	Standard deviation	Mice with tumors	Mean latent period in days/30	Standard deviation	Mice with tumors	Mean latent period in days/30	Standard deviation
		Number			*Number*			*Number*		
0.25	−0.602	20	2.37	0.38	18	3.60	0.74	17	5.62	1.15
0.5	−.301	20	2.22	.44	19	3.22	.65	18	5.45	1.06
1.0	.000	20	2.13	.40	20	2.90	.42	19	5.06	1.06
2.0	+.301	20	2.36	.42	20	2.97	.53	17	4.14	.95
3.0	+.477	.....	.....	.....	.....	.....	.....	15	3.84	.69

which are plotted in figure 2. An analysis of the variance [8] of the latent periods is shown in tables 4 and 5. It was found that the variation between dose groups was significant ($F=7.08$; $P<0.01$) and that most of the variation between groups could be accounted for by a linear regression ($F=2.08$; $P>0.05$). The ratio between the mean squares (F, table 5), though not sufficiently high to indicate a significant departure from a linear regression, nevertheless suggests that some other curve might better account for the regression. Examination of the original data and of figure 2 indicates that the slight deviation is due almost entirely to the responses of the highest dose group. It is possible that the minimal latent time was reached with the two highest doses and that further increase in dose failed to reduce the response as in the preceding experiment with methylcholanthrene. This question, however, can be answered only by further experimentation.

TABLE 4.—*Analysis of variance of latent periods*

[Benzpyrene in mice]

Source of variation	Degrees of freedom	Sum of squares	Mean square
Between dose groups	3	5.589	1.863
Within dose groups	73	19.197	.263
Total	76	24.786	

$$F=\frac{1.864}{0.263}=7.08;\ P<0.01$$

Data from Shimkin and Andervont (29).

TABLE 5.—*Analysis of variance for testing goodness of fit of regression line*

[Benzpyrene in mice]

Source of variation	Degrees of freedom	Sum of squares	Mean square
Linear regression	1	4.493	4.493
Deviation from linear regression	2	1.096	.548
Within dose groups	73	19.197	.263
Total	76	24.786	

$$F=\frac{0.548}{0.263}=2.08;\ P>0.05$$

Data from Shimkin and Andervont (29).

[8] *In these analyses use was made of tables of the F statistic given by Snedecor (30).*

The data obtained with dibenzanthracene show a highly significant variation between the responses at different dose levels. The original data are summarized in table 3 and figure 2. An analysis of the variance of the latent periods is given in table 6. The value of F in this case was 10.25, and the variation between doses is highly significant ($P<0.01$). That most of the variation between doses can be explained by a linear regression of the responses on log dose is indicated by a further analysis of the variance as indicated in table 7. The deviations from a linear regression are not significant ($F=0.98$; $P>0.05$), and there is no evidence to indicate that some other type of curve would describe the present data better than the linear relation which has been assumed. It may be concluded, therefore, that in this experiment the relation between mean latent period and the logarithm of dose is satisfactorily described by a straight line. The equation to the calculated regression line was found to be

$$Y=4.863-1.748\ (x+0.039)$$

where Y represents the expected value of the mean latent period in days/30 corresponding to an assigned value of x, or dose in log milligrams.

TABLE 6.—*Analysis of variance of latent periods*

[Dibenzanthracene in mice]

Source of variation	Degrees of freedom	Sum of squares	Mean square
Between doses	4	41.420	10.355
Within doses	81	81.827	1.010
Total	85	123.247	

$$F=\frac{10.355}{1.010}=10.25;\ P<0.01$$

Data from Shimkin and Andervont (29).

TABLE 7.—*Analysis of variance for testing goodness of fit of regression line*

[Dibenzanthracene in mice]

Source of variation	Degrees of freedom	Sum of squares	Mean square
Linear regression	1	38.458	38.458
Deviation from linear regression	3	2.956	.986
Within doses	81	81.827	1.010
Total	85	123.241	

$$F=\frac{0.986}{1.010}=0.98;\ P>0.05$$

Data from Shimkin and Andervont (*29*).

Data of Lettinga

The investigation of Lettinga (*28*), which was used in the previous section on percent response, also included detailed information on the latent period of the tumors obtained in the different dose groups. The doses, with the average latent-period responses and standard deviations, are summarized in table 8.

TABLE 8.—*Summary of data of Lettinga (28) showing mean latent periods and standard deviations obtained at various dose levels*

[Dibenzanthracene in mice]

Total dose		Mice with tumors	Mean latent period, in days/30	Standard-deviation
Milligrams	Log milligrams			
		Number		
5.0	0.699	5	4.30	1.14
2.5	.398	8	3.80	.56
1.0	.000	9	3.57	.44
0.5	−.301	10	3.61	.61
0.25	−.602	26	4.57	1.63
0.125	−.903	9	5.94	1.12
0.05	−1.301	4	7.33	.80
0.0125	−1.903	4	8.07	1.18
0.005	−2.301	0		

Analysis of the total data shows that the relation between latent-period response and log dose is not satisfactorily described by a linear regression. Inspection of figure 3, in which the observed points are plotted, reveals that this is largely owing to a change in slope, or a break in the response curve, at about log dose −0.301 (0.5 mg.). The curve at this point flattens out, and

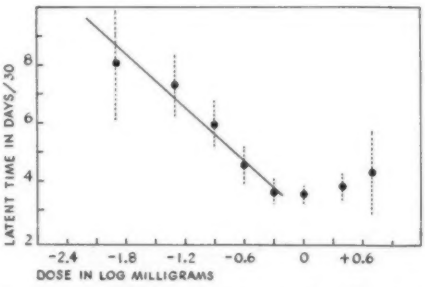

FIGURE 3.—Dose-response curve for dibenzanthracene in mice, showing regression of latent period on log dose.

Data of Lettinga (*28*).

Vertical broken lines: limits of error for plotted means ($P=0.05$).

the latent period is not further decreased by any of the successively higher doses. If anything, the response trends in the opposite direction, but this trend is not statistically significant. This phenomenon is probably due to the same factors described previously for the results with methylcholanthrene, i. e., there is a minimal latent time below which tumors do not appear regardless of increases in dose.

The upper limit of the significant dose range was set at −0.301; therefore, the remainder of the latent-period data was analyzed for its relationship with log dose and its goodness of fit to a linear regression. The anlaysis of responses between log dose −1.903 and −0.301 is given in tables 9 and 10. The variation between doses was found to be highly significant ($F=12.86$; $P<0.01$) and could be accounted for by a linear regression ($F=0.75$; $P>0.05$). The equation to the calculated regression line was found to be

$$Y=5.094-3.055\ (x+0.747).$$

The line is shown graphically in figure 3. It is extended to the smallest dose employed by Lettinga, namely, −2.301 (0.005 mg.). This dose failed to produce

tumors in any of 20 test animals [9] while the next highest dose, −1.903 (0.0125 mg.), gave only 4 tumors in a total of 20 test animals. For practical purposes, then, it may be considered that the entire significant range within which latent period is correlated with dose was covered by Lettinga and that the relation does not depart significantly from a straight line within this range. Actually, it is seen, a slight curve would fit all of the observed means better than the straight line which has been drawn. This alignment of means could have occurred due to chance, however, and there is no indication that a curvilinear relation would better describe the regression in this instance.

TABLE 9.—*Analysis of variance of latent periods*

[Dibenzanthracene in mice]

Source of variation	Degrees of freedom	Sum of squares	Mean square
Between doses	4	91.044	22.761
Within doses	48	84.957	1.770
Total	52	176.001	

$$F=\frac{22.761}{1.770}=12.86;\ P<0.01$$

Data from Lettinga (*28*).

TABLE 10.—*Analysis of variance for testing goodness of fit of regression line*

[Dibenzanthracene in mice]

Source of variation	Degrees of freedom	Sum of squares	Mean square
Linear regression	1	87.055	87.055
Deviation from linear regression	3	3.989	1.330
Within doses	48	84.957	1.770
Total	52	176.001	

$$F=\frac{1.330}{1.770}=0.75;\ P>0.05$$

Dataf rom Lettinga (*28*).

[9] *Dobrovolskaia-Zavadskaia* (27) *obtained only 13 tumors in 364 treated animals, an incidence of 3.57 percent, with the same dose (cf. table 1).*

Comparison of Results of the Two Studies

The response curves calculated from the data presented in the two preceding sections are compared in figure 4. The data of Lettinga (*28*) have been considered only in the range of doses where a correlation was obtained between dose and response. The arrow at the right extremity of the lower curve shows the minimal latent period obtained by Lettinga. It will be observed that the two curves do not differ greatly in slope although the difference is probably significant ($P=0.04$). On the other hand the difference between the relative positions of the two curves is highly significant ($P<0.01$).

It is impossible to assign specific meaning to these differences since the solvent as well as the technique of administration was different in the two studies. Furthermore, it is probable that the test animals used in the respective studies were not of the same genetic constitution. Although the strain employed by Lettinga is not given, he does state that his stock was susceptible to the development of pul-

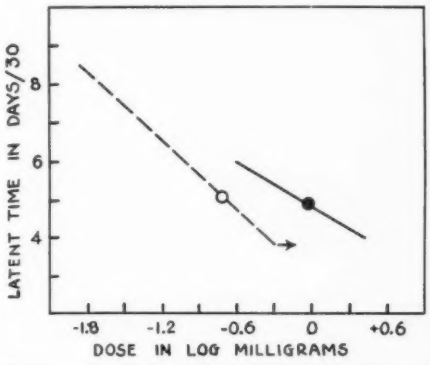

FIGURE 4.—Comparison of dibenzanthracene response curves of figures 2 and 3.
Upper curve: data of Shimkin and Andervont (*29*).
Lower curve: data of Lettinga (*28*).

monary tumors which is not the case for the C3H stock employed by Shimkin and Andervont (*29*). An additional factor which could have contributed to the difference in position in the response curves is a difference in biological potencies of the two preparations of dibenzanthracene, particularly due to variation in the amount of impurities present.

It may be concluded that within the dose range investigated the relationship between latent period and the logarithm of quantity of dibenzanthracene is satisfactorily described by a straight line. Methods for the analysis of linear dose-response data (pt. I) are therefore applicable to results obtained with this material in mice. Similar findings were obtained in the analysis of data on benzpyrene.

PART III: APPLICATIONS TO SPECIAL PROBLEMS WITH CARCINOGENIC HYDROCARBONS

It was demonstrated in the preceding section that results obtained with carcinogenic agents are subject to expression in the form of linear dose-response curves and that the established procedures for dealing with this type of relation are applicable to the quantitative analysis of data obtained with these compounds.

Four types of problems may be recognized for a consideration of the practical applications of the methods to investigations with carcinogenic agents. They are as follows: (1) Determination of the limits of sampling variation of the biological response to known quantities of a given agent (i. e., error of the response curve); (2) estimation of the relative potencies of various preparations of a given agent by comparison with a standard preparation of the same material on a basis of biological responses (i. e., bioassay); (3) comparison of the relative carcinogenic potencies of different chemical carcinogens; and (4) application of the standard dose-response relation and information pertaining to it to the solution of specific questions encountered in the planning and execution of experiments. Problems of the first two types are considered only briefly since the principles of these analyses have been described in detail by others (*7*, *8*, *12*, *21*, *22*).

The Sampling Error of the Response Curve

When the purpose of an experiment is to establish the response of a given animal population to known quantities of a given agent, it is desirable to know within what limits of error the response has been estimated.[10]

Since the variations in results with carcinogenic agents follow the same general trends which have been found in the instance of numerous other types of biological materials, the established statistical procedures for determining the errors of response curves are applicable to data obtained with carcinogenic hydrocarbons. The general statistical procedures involved have been described in detail by Gaddum (*7*), Bliss (*8*, *22*), and Irwin (*12*) for the quantal type of response, and by Irwin (*12*) for the measurable response.

It should be pointed out that certain of the general procedures may have to be modified to suit the particular data under consideration in order to reach accurate estimates of the limits of variation and of the significance of results. In the case of carcinogenic hydrocarbons, the standard deviation of latent periods appears to be correlated with the logarithm of dose; i. e., the standard deviation appears to be greater at lower doses of the carcinogen than at higher doses. The

[10] *See curved lines of figs. 1 and 10.*

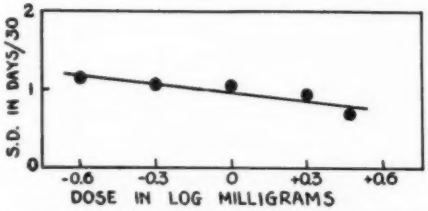

FIGURE 5.—Standard deviation of latent periods with successive doses of dibenzanthracene in mice. Data of Shimkin and Andervont (*29*).
Circles: observed standard deviations.
Solid line: calculated regression line.

regression is slight, however, and with data available at present cannot be proved to be significant. The standard deviations of tables 3 and 8, derived from results with dibenzanthracene in mice, are plotted against log dose in figures 5 and 6. The standard deviations of figure 5 (Shimkin and Andervont) appear to follow a definite trend, but the variation between doses is not significant. Observations on a larger number of animals at these doses or observations with wider spaced doses, however, might be expected to show a significant regression. The data of Lettinga (fig. 6) show a significant variation between doses, but it is greater than can be accounted for by the regression alone. This is believed to represent heterogeneity among the test animal groups, although it is possible that other factors may be involved.

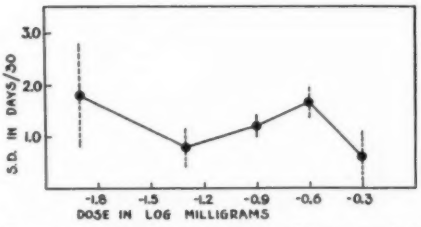

FIGURE 6.—Standard deviation of latent periods with successive doses of dibenzanthracene in mice. Data of Lettinga (*28*).
Circles: observed standard deviations.

Another question which arises in connection with the analysis of data obtained under specific conditions is the nature of the frequency distribution (e. g., latent periods) at various dose levels. Sufficient data are not available for a separate analysis at different dose levels, but the deviations have been expressed as standard deviation units and the data of all doses in a given experiment combined to give the distribution curves of figure 7.

It is noted that the results are similar for both the data of Lettinga and of

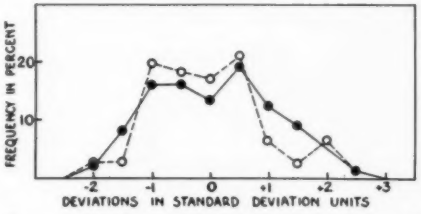

FIGURE 7.—Frequency polygons showing variations of latent periods with dibenzanthracene in mice. Combined data of all dose groups expressed as deviations from group means in terms of standard deviation units.
Broken line: data of Lettinga (*28*).
Solid line: data of Shimkin and Andervont (*29*).

Shimkin and Andervont. Whether the bimodal feature of these curves is characteristic for dibenzanthracene in mice, or whether it is an artifact due to combination of observations or to experimental conditions cannot be stated at present. It emphasizes the fact that for quantitative work it is essential to employ standard conditions with regard to animals as well as to laboratory procedures. Since the latent period may be related to such factors as age, or weight (*5*), and sex (*31*), it is necessary that these sources of variation be adequately controlled in establishing the standard response curve and the nature of its variation. With carcinogenic hydrocarbons, another source

of variation may be encountered in the type of tumor which is produced at the site of injection. It is known that a small percentage of the subcutaneous tumors in C3H males, for example, are squamous-cell carcinomas rather than sarcomas (*29*), and that subcutaneous sarcomas in mice may be of several histological types (*32*). It is quite possible that the latent period may be different for fibrosarcomas and for myosarcomas, or for mixed tumors as contrasted with homogenous tumors. This source of variation has not been considered sufficiently and must await further experimental data.[11]

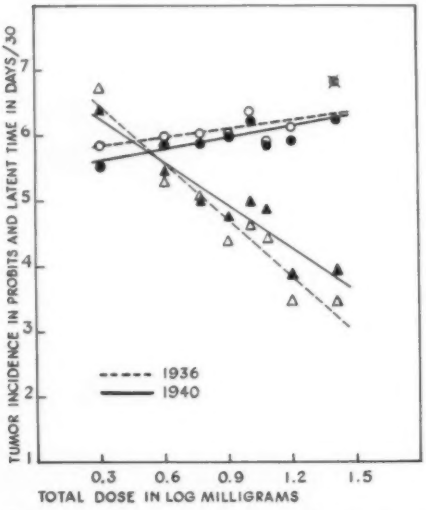

FIGURE 8.—Regression of latent-period responses and probit responses on log dose for benzpyrene in rats, showing reproducibility of dose-response curves at a 4-year interval. Data of Dunning and Curtis, et al. (*33, 34*).

Open circles: probit responses, 1936.
Closed circles: probit responses, 1940.
Open triangles: latent period responses, 1936.
Closed triangles: latent period responses, 1940.

[11] *Studies directed toward this end and toward the establishment of response curves over a wide range of doses are now in progress at the National Cancer Institute.*

BIOASSAYS

The problem of bioassay is at the present time of secondary interest in its relation to pure chemical carcinogenic compounds because physical and chemical methods are available for assaying these materials.

If various preparations of a given agent differ only in the concentration of the specific agent which they contain, that is, if they differ only in potency, the slope of the response curves will be constant (within limits of random variation) for all preparations provided the animal population is uniform. In the instance of a given agent, therefore, it is possible to compare various preparations of it on a basis of the respective quantities required in each case to produce a common animal response. The comparison may be made at any level of response since the response curves will be parallel lines separated by horizontal distances equal to the logarithms of the potency ratios of the materials which they represent.

If it can be demonstrated that the sensitivity of the animal population remains relatively stable over long periods of time, the slope of a previously determined standard response curve as well as its errors of estimate may be used to advantage in subsequent studies involving estimates of potencies and their standard errors. Encouraging evidence is found in the results of Dunning, Curtis, and their coworkers (*33, 34*) that both the slope and the position of response curves obtained with benzpyrene in rats are relatively stable over a period of several years. These investigators studied responses to two concomitant controlled variables, the total dose and the number of injection sites. The results obtained under those particular conditions were essentially the same in two studies reported 4 years apart. The observed responses and calculated regres-

sion lines obtained in their separate studies are shown in figure 8.

The statistical procedures involved in the problem of bioassay are presented in detail by Gaddum (*7*), Bliss (*21*), and Irwin (*12*). References to specific applications of the procedures have been cited in the Introduction.

Comparison of Carcinogenic Potencies of Different Hydrocarbons

In the instance of different agents the respective response curves are not necessarily parallel even with comparable test animals, as is the case with various preparations of a single agent. When the slopes are thus different, the horizontal distance between the response curves is not constant but different for each level of response. Widely differing conclusions regarding relative potency might therefore be reached depending upon the level of response at which the comparison is made. If different agents are to be compared with regard to relative carcinogenic potencies, it is obvious that some particular level of response must be chosen as a point of reference. It is further obvious that any comparison made in this way, that is, by reference to a standard response, would be entirely relative and referable only to the particular response involved. It is desirable, therefore, that a point of reference be chosen which has a common significance for all response curves which are to be compared.

A point on the dose-percent response curve which has a common significance in the case of different agents regardless of variations in the slopes of the curves is the point corresponding to the 50-percent response. On the transformed probit response curve this point is represented by 5.0 probits. The 50-percent point (or 5.0-probit point) corresponds to the median or average amount of material which just causes the biological response in the particular population of animals involved, that is, it represents the median effective dose. Differences in the slopes of the response curves for different agents would indicate differences in the variation of individual effective doses, but the median effective dose has the same significance in all cases.

The potency of any given material can be expressed in terms of the amount, e. g., milligrams, required to give a 50-percent response. Thus, if one material is twice as potent as another, it will produce a 50-percent response with half of the amount required for the second compound. When the response curve is considered in linear form, the point at which it crosses the ordinate corresponding to 5.0 probits represents an estimate of the logarithm of the 50-percent-point dosage.[12] The antilog of this value, therefore, represents the median effective dose. This may be designated by the symbol ED_{50}, as suggested by Foster (*10*). Since the quantity of a given material represented by ED_{50} is that quantity required to give a fixed biological response (i. e., 50 percent) this quantity may be considered as 1 biological unit, or, in this case as 1 carcinogenic unit. The ratio of the number of milligrams per carcinogenic unit, therefore, represents the potency ratio of the materials being compared.

In the routine determination of ED_{50} it is not usually practical to employ large numbers of test animals. Bliss (*22*) has described the procedures for determining

[12] *Gaddum* (7) *has presented and discussed the relative merits of several different procedures which have been suggested for the calculation of the median effective dose.*

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the response curve as well as ED_{50}[13] when small numbers of test animals are involved. Estimation of log ED_{50} from the calculated probit response curve is illustrated graphically in figure 9. Line A of this figure represents the probit-dose data of Lettinga for dibenzanthracene in mice (cf. table 2 and fig. 1). The vertical arrow has been drawn to intersect the line at the point opposite 5.0 probits. The logarithm of ED_{50}, indicated by the base of the arrow, is found to be -1.3 and ED_{50} is the antilog of this value, or 0.05 mg. The equation to this regression line, namely,

$$Y_{(\text{probits})} = 5.336 + 1.439\ (x + 1.070)$$

may be rearranged and solved for x, when $Y = 5.0$, to give the log dose (x) corresponding to 5.0 probits, to determine log ED_{50}. Thus

$$\text{Log}\ (ED_{50}) = \frac{5.0 - 5.336}{1.439} - 1.07 = -1.303,$$

and the antilog, or ED_{50}, is 0.0498 mg. The standard error of log ED_{50} was found by use of Bliss' equation 20 (*22, p. 202*) to be ± 0.086. Log ED_{50} is, therefore, -1.303 ± 0.086, and ED_{50} is $0.0498\ ^{+0.010}_{-0.008}$, or the error expressed as an average, 0.0498 ± 0.009.

Unfortunately, data are not available for determining the ED_{50} of other carcinogenic hydrocarbons in mice. The data of Shimkin and Andervont (*29*) for three different hydrocarbons were obtained with doses which gave practically 100-percent responses in all cases, and it is therefore impossible to obtain information from their data relative to the percent response curve.[14]

[13] *The notation LD_{50} (median lethal dose) was used by Bliss since the biological response was death. Foster (10) suggested an appropriate change in the first term of the general expression ED_{50} (median effective dose) to suit the particular biological effect being studied, as ND_{50} where the effect is narcosis. In keeping with this precedent the expression TD_{50} (median tumor dose) might be employed in connection with tumor-producing agents.*

[14] *See footnote* 11.

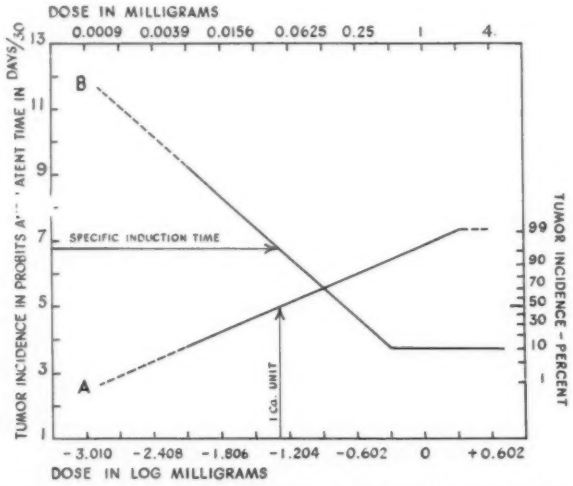

FIGURE 9.—Dose-response curve for dibenzanthracene in mice, showing regression of probit responses and latent period responses on log dose. Data of Lettinga (*28*).

A: probit response curve.

B: latent-period response curve.

The right and upper scales give equivalent values in percent and arithmetic dose, respectively.

Insofar as we have been able to determine, there is no point on a latent-period response curve that has a common significance in the case of different hydrocarbons. The latent-period data, however, may be combined advantageously with the percent data in order to express the relative biological activities of different carcinogenic agents. For example, from the latent-period response curve it is possible to estimate the average time at which tumors appear at the dose level corresponding to ED_{50}. The latent period in this case has a special significance in that it measures the rate of action of a particular biological unit of a given substance (*1 carcinogenic unit*). For the purpose of further reference this particular latent period may be designated as the *specific induction time.*

The determination of the specific induction time from the latent-period response data of Lettinga (*28*) is illustrated graphically in figure 9. Curve B of this figure represents the latent-period response curve drawn on the same dose scale as the probit response curve A. The horizontal arrow intersects the latent-period curve at the dosage corresponding to ED_{50}, i. e., at the dose level indicated by the vertical arrow of this figure. The base of the horizontal arrow indicates the average time, in days/30, at which tumors would be expected to appear with dose ED_{50}. This time, which in the present instance is 6.8 days/30, represents the specific induction time.

The equation to the latent-period response curve, namely,

$$Y_{(\text{days}/30)} = 5.094 - 3.055\ (x + 0.747),$$

also may be used to determine the specific induction time in days/30 by substituting for x the value of log ED_{50} and solving for Y as follows:

$$Y_{(\text{days}/30)} = 5.094 - 3.055\ (-1.303 + 0.747) = 6.79 \text{ days}/30.$$

The standard error of the specific induction time, which is calculated from a fitted response curve, may be determined from equation 37 given by Irwin (*12, p. 19*). The standard error in this instance is ± 0.23, and the specific induction time is 6.79 ± 0.23 days/30.

The specific induction time may be employed with the ED_{50} to show, simultaneously, the speed of tumor production with, and the total quantity of an agent required to give 1 carcinogenic unit.[15] Thus, the response of a series of carcinogens can be represented by plotting graphically the specific induction time against ED_{50}. For example, it is possible that two materials might have the same ED_{50} but might differ in relative speed of action. In such a case the two points would fall in the same vertical plane on the dosage scale but would differ in position according to their specific induction times. Contrariwise, two materials having the same specific induction time, thus falling in the same horizontal plane, may differ in the quantity of material composing the ED_{50}. If these two indicators of biological potency, the quantity and the intensity factors, are correlated in a systematic manner for different carcinogenic compounds, it is possible that some combined mathematical function or relation may be employed to

[15] *Fieser* (4) *suggested the "maximum effective dose," the dose which produces the greatest incidence of tumors in the shortest possible time, as a measure of potency of carcinogenic hydrocarbons. This dose is represented in figure 9 by the value of the abscissa corresponding to the point at which the latent-period response curve breaks and continues as a horizontal line. Although this measure may prove to be of value, there is no evidence at the present time to indicate that it is satisfactory for the comparison of relative potencies of carcinogens. It is probable that the chief limiting factor that would be encountered in the use of this method would be the practical doses of the materials which are to be compared, both from the standpoint of availability of the agents and the ability of the animals to tolerate such amounts.*

advantage.[16] Such a procedure, however, is not advisable until the nature of the correlation is known.

Specific Questions

The standard dose-response data can be applied to certain practical problems which are encountered during the course of experiments with carcinogenic compounds, and to the planning of further investigations with these materials. The following discussion is pursued in the form of answers to specific questions. It is immediately apparent that these questions can be answered only as they apply to particular concrete findings of actual previous experimentation in which dose-response relations have been established under standard conditions with regard to the agent, the technical procedures, and the test-animal population.

The accuracy with which the questions can be answered, of course, depends upon the nature as well as the quantity of standard dose-response data available for the analysis. Further accumulation of data may necessitate certain modifications of the procedures and of the actual values represented here, but the present data may be used to illustrate the principles as well as to serve as a basis for the planning of future studies.

How Long Should Animals be Observed in Order to Determine the Percentage of Tumors Elicited?

It is of considerable importance to be able to predict the time limits of an experiment, in order that the observations might not be terminated before the test animals have had a maximum opportunity to develop tumors and, conversely, in order not to maintain animals for prolonged periods after they have had a maximum chance to develop tumors.

[16] *Such as Iball's* (25) *carcinogenic index.*

Previous knowledge regarding the latent-period response curve and the nature and extent of variations in individual responses at different dose levels provide a basis for setting a time limit for the appearance of tumors at any given dose level.

In the absence of precise information regarding the nature of the frequency distribution and the standard deviation, S, about the calculated regression line, the present procedure may be illustrated by use of tables of the probability integral pertaining to the normal curve and the value of S estimated on the assumption that it is constant at all dose levels.[17]

By considering the practical time limits beyond which no tumors would be expected to appear to be those limits corresponding to $\pm 1.965 \times S$, a chart (fig. 10) was prepared from the data on dibenzanthracene in C3H male mice (Shimkin and Andervont) to show the time limits, both above and below the calculated regression line, which correspond to these deviates. The highest and the lowest lines of this figure show, respectively, the times above and below which the expected frequency of tumors is only 25 in 1,000 or less, and therefore negligible for practical purposes. The highest line shows for the various dose levels the minimal time that observations should be continued in order for all animals to have a maximum opportunity to develop tumors. The accuracy with which this analysis predicts the result for the population as a whole cannot be stated from this one experiment, but the limits within which

[17] $S = \sigma\ (1 - r^2)$, *where* σ *represents the standard deviation about the group mean of the series, and* r *is the correlation coefficient. Although the evidence indicates that the observed standard deviations are slightly increased as the dose is decreased, this does not necessarily mean that the standard deviation about the calculated regression line will differ appreciably at different dose levels. Further studies involving several separate experiments are necessary to clarify this point.*

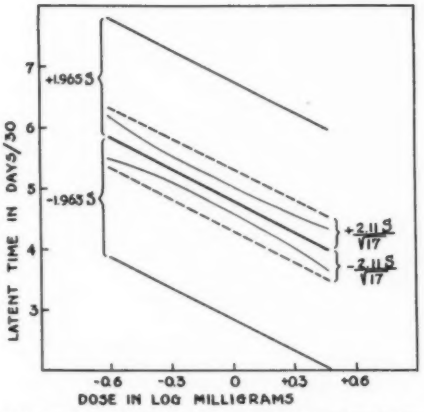

FIGURE 10.—Line chart showing limits of variation of latent periods about calculated regression line for dibenzanthracene in mice. Data of Shimkin and Andervont (*29*).

Upper and lower lines: limits which include individual latent periods ($P=0.05$). The upper line indicates time required for maximum opportunity to develop tumors.

Broken lines: limits which include means based on 17 observations.

Heavy middle line and curved lines: calculated regression line and limits of error ($P=0.05$), respectively.

the true results might be expected to fall can be estimated. These are indicated by the curved lines on either side of the calculated regression line which represent the limits of error of the regression line corresponding to $P=0.05$.

The limits of variation (corresponding to $P=0.05$) of mean responses based on the average number of animals at each dose, 17, are included in figure 10. They are represented by the dash lines.

How Should Animals Dying During the Course of an Experiment Be Considered in the Determination of Percent Response?

Experiments with carcinogens which extend over a considerable length of time usually entail some extraneous mortality among the animals. There is no difficulty of interpretation if all the animals injected with a compound develop tumors, or if the animals which do not develop tumors die of other causes at a prolonged period after the individuals which did develop tumors.

When animals die during the course of an experiment designed to determine tumor incidence, the incidence of tumors can no longer be interpreted ideally. Any method devised to compensate for this unavoidable source of interference is at best only a method of obtaining an approximation to the actual results which would have been obtained if no animals had died.

When information concerning the frequency with which tumors appear after various time intervals among animals treated with a given carcinogenic agent is lacking a rectangular distribution extending over the entire period of observation might be assumed and each animal dying without a tumor during the observation period might then be considered in relation to the fraction of the total time during which it was alive. The error introduced by extraneous mortality during the experiment may also be corrected to a certain degree by the use of the "effective total" procedure of Fieser (*4*), which excludes all animals dying before the advent of the first tumor in a given test group but includes all which die after this minimal time.

A better approximation to the true tumor incidence can be made, however, if the frequency of tumors at various time intervals is accurately known. Animals dying without tumors during the course of an experiment can then be "weighted" according to the proportion of animals, within the tumor population, which had developed tumors at or prior to the time of death of the particular animal under consideration. The principle of this procedure is to judge (or "weight") the

animals that die without tumors on a basis of the behavior of the animals which do develop tumors. If the number of animals in a given group is fairly large, the frequencies of tumors at various time intervals may be estimated satisfactorily from the data of this group alone and appropriate weighting factors may be determined for evaluating the animals which died without tumors during the course of the experiment. The data of Shimkin (*24*) with methylcholanthrene in male mice of strain L may be used to illustrate the procedure. In the experiment concerned, each of 83 mice was injected subcutaneously with 0.2 mg. of methylcholanthrene dissolved in tricaprylin. Fifty-one of this number eventually developed tumors, while 20 dying during the course of the observations and 12 living throughout the experiment failed to develop tumors. The 51 animals developing tumors may be used as a basis for determining the significance of the 20 which died during the experiment. For this purpose an accumulation of the tumor frequencies, in percent, is plotted against time as shown in figure 11.[18] The smoothed curve drawn through the plotted points may be used to estimate the percent of the total group of tumor animals which would be expected to develop tumors at or prior to any given time represented on the abscissa scale. The percentage values expressed as decimal fractions, i. e., proportions of unity, may be used as weighting factors. Thus, at 15 weeks after injection only 28.0 percent of the tumor population would be expected to have developed tumors. An animal dying at this time would, therefore, be given a weighting factor of 0.28. Since the uncorrected "weight" of each individual is unity, the "weight" of this particular animal in the final corrected total would be 0.28×1, or 0.28. Table 11 shows the time of death of each animal as determined from the original data of Shimkin. The weighting factors determined from figure 11 are given in column 4. The calculations of the corrected total and the tumor incidence are also illustrated in this table.

[18] *This figure represents a reproduction of the left-hand curve of Shimkin's figure 3* (24).

TABLE 11.—*Determination of "corrected total" of animals dying during observation period*

Animal categories	Time of death after injection (days/30)	Number of mice	Weight factor [1]	Corrected weight
Mice developing tumors	.....	51	1.00	51.00
Mice living throughout experiment, but failing to develop tumors	.....	12	1.00	12.00
Mice dying without tumors during course of experiment	2.30	2	.02	.04
	2.80	2	.12	.24
	3.03	1	.16	.16
	3.97	5	.49	2.45
	4.43	1	.73	.73
	4.67	1	.80	.80
	4.90	2	.88	1.76
	5.37	1	.96	.96
	5.60	1	.97	.97
	6.07	1	.98	.98
	6.30	2	.99	1.98
	6.53	1	1.00	1.00
Total	.....	[2] 83	.....	[3] 75.07

[1] See text.
[2] Original.
[3] Corrected.

$$\text{Tumor incidence} = \frac{51}{75.07} \times 100 = 67.9 \text{ percent}$$

Data from Shimkin (*24*).

If the total number of animals developing tumors in a given experiment is small, the frequency curve determined from the data of the experiment alone may be subject to considerable error and hence unsatisfactory for judging the significance of animals that die without tumors. Where the sensitivity of the animal population is stable over long periods of time, it is possible to employ previously determined standard dose-latent period data (cf. pt. II) as a basis for estimating the significance of animals that die in subsequent experiments involving only small numbers of animals, provided of course that the standard relations have been

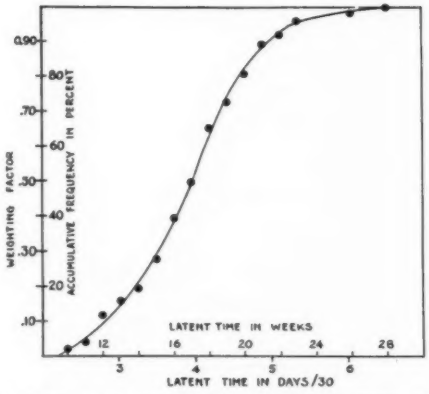

FIGURE 11.—Accumulative frequency curve of latent-period responses to 0.2 mg. of methylcholanthrene in strain L male mice. Data of Shimkin (*24*). Decimal fractions represent weighting factors for animals that die without tumors at times indicated by corresponding points on the curve.

established with sufficient accuracy. For this purpose, the frequency curves may be determined separately at each dose level by the procedure described. Or, if the trends of the mean responses and of the standard deviations are uniform over a wide range of doses, the respective regression equations may be employed to construct a line chart for reading directly the expected frequencies of tumors (and thus the weighting factors) at any dose level and after any time interval within the range represented.

A line chart of this sort (fig. 12) was prepared for determining the weighting factors used in deriving the corrected totals employed in the analysis of the percentage data of Lettinga (table 2). In this experiment the number of animals with a tumor in most of the dose groups was too small for a separate analysis in each case. Although the trend of the observed standard deviations was somewhat irregular, an average trend, represented by the best straight line through the observed values, and tables of the probability integral, pertaining to the normal curve, were used to obtain an estimate of the animals that died during the period of observations. The corrected totals thus derived are not emphasized as exact, but they are based on the trend of the data as a whole and are probably as representative as any estimates that could be made under the conditions.

How Many Animals Should be Employed at the Various Dose Levels to Give Observations Having the Same Degree of Reliability?

If the variations about the calculated regression line do not differ significantly at different dose levels, the same reliability of results will be obtained with a given number of significant animals regardless of the dose employed. By significant animals is meant those which actually contribute

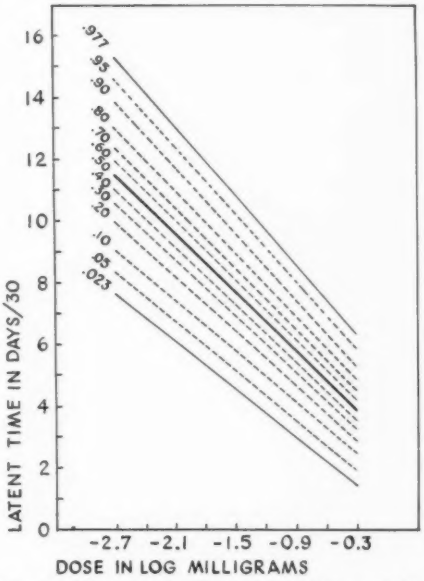

FIGURE 12.—Line chart for determining weighting factors for animals that died during the course of Lettinga's experiment (*28*) with dibenzanthracene in mice. See text.

to the results—animals which develop tumors in the case of latent-period observations, or the corrected total numbers of animals employed in determinations of the percent response.

Although the variations of latent periods may show a slight regression on log dose, the differences encountered within the practical experimental range of doses probably will not be great enough to cause serious differences in reliability at different levels, provided the number of significant animals is essentially the same in all cases. The studies of Lettinga (*28*) and of Dobrovolskaia-Zavadskaia (*27*), however, show that the percentage of treated animals which develop tumors decreases systematically as the dose is decreased. Thus, for a given number of animals treated, the number available for studies on the latent period will be smaller at smaller doses than at larger doses. In order to estimate the number that should be treated originally, so that the number of animals with tumors will be practically the same at all levels, it is necessary to have information concerning the expected frequencies of animals with tumors at various dose levels. For this purpose a previously determined standard dose-percentage-response curve may be used. The transformed dose-probit curve used in other analyses may be made available for this purpose by the addition of percentage equivalents of the probit values as indicated along the right ordinate scale of figure 9.

The reliability of an estimated probit value in addition to its relation to the number of observations is also dependent in part upon the magnitude of the probit value itself. The relative influence of the latter factor is fixed for any given probit value and may therefore be calculated once and for all (*7*, *8*). A table showing the relative "weights" as well as the relative numbers of animals required to give equal weight at successive probit levels has been published by Bliss (*8*, *table III*). By establishing the doses of a given hydrocarbon required to give percentage responses corresponding to various probit values, it is possible by use of Bliss' table to estimate the relative numbers of animals required at various dose levels to yield observations of equal weight. The procedure is illustrated in table 12, by using the data of Lettinga on dibenzanthracene in mice. The ideal probit values were calculated from the regression equation given in part II. The ideal percentage equivalents were determined from the calculated probits by use of Bliss' table I (*8*). The relative number of animals is given in terms of the number required when 10 are used at the point of greatest reliability, namely, at 5.0 probits.

TABLE 12.—*Relative number of mice required at different dose levels to give probit responses of equal weight*

[Dibenzanthracene in mice]

Dose in milligrams	Dose in log milligrams	Ideal tumor incidence		Relative weights of responses	Relative number of mice for equal weight
		Probits	*Percent*		
2.0	−0.301	7.309	98.95	0.075	86
1.0	.000	6.876	97.9	.160	40
0.5	−.301	6.443	92.6	.288	22
0.25	−.602	6.010	84.4	.402	16
0.125	−.903	5.577	78.1	.563	12
0.0625	−1.204	5.144	55.7	.631	10
0.0312	−1.505	4.710	38.6	.617	10
0.0156	−1.806	4.277	23.5	.525	12
0.0078	−2.107	3.844	12.4	.385	16
0.0039	−2.408	3.411	5.6	.241	27
0.0019	−2.709	2.978	2.15	.126	51
0.0009	−3.010	2.545	.70	.044	146

Data from Lettinga (*28*).

The actual number of animals required for specific purposes, of course, will depend upon the aim of the observations and upon the degree of accuracy required. From data available at present, it is not possible to determine with confidence the number of animals that would be actually expected to give a certain predetermined degree

of accuracy. It may be stated, however, that on the basis of present data the number required at the levels of the respective mean responses to give estimates of potency within ± 0.3 of a logarithmic interval (i. e., $^{+100\text{ percent}}_{-50\text{ percent}}$ in terms of arithmetic dose) is approximately 100 animals.

CONCLUSIONS

It is evident from the examples presented in full, as well as from other data which have been analyzed by these techniques, that the biomathematical methods are applicable to the expression of results with carcinogenic materials.

It must be emphasized that these methods express biological phenomena; they contribute to the interpretation of the phenomena in that they clarify relationships. For example, the presentation of data by these methods cannot in itself elucidate the mode of action of the agents. Moreover, although the methods can and do visualize the accuracy and the reproducibility of the experimental results, they cannot be any more accurate than the facts upon which they are based. Thus, the representation of results by these methods does not supplement, but complements the presentation of the specific findings.

The use of the method presupposes certain basic information concerning the stimulus to be tested, and the organism to be used as the test animal. This basic information, however, should be obtained with the view of making it susceptible, after further elaboration, to analyses by biomathematical methods. In the first place, therefore, the experiment should be as free of avoidable sources of variation as possible, in order that the known variables may be revealed as accurately as possible. The bioassay techniques which have been found useful in previous work with carcinogenic hydrocarbons and which have been found to be consistent, are reviewed elsewhere. Many compounds which have been tested for local carcinogenic potency in the mouse were administered by procedures which permitted only a gross estimate of the dose, and only one dose was employed. Since the response curve is the foundation of the biomathematical approach, it is evident that the use of several exact doses covering a wide range is necessary for the application of these methods.

This presentation was stimulated by the fact that these biomathematic methods have been found to be not only desirable but also indispensable in quantitative experiments with a wide range of biologically active agents, and by the possibility that a similar service would be rendered by the application of these methods to problems in cancer research. It is felt that the thesis has been fully justified. In summary, it may be stated that four advantages accrue from the use of the biomathematical procedures in research with carcinogenic materials: (1) The presentation of the experimental data in a clear and concise manner; (2) the estimation of the statistical significance and reproducibility of the data; (3) the interpolation of data, within the range of the observed results, without further actual experimentation; and (4) the guidance of further experimentation by the extrapolation of the available data. The interpretation of the known results is thus facilitated, and the planning of further experimentation is made much more certain of supplying the missing information.

REFERENCES

(*1*) Cook, J. W., Haslewood, G. A. D., Hewett, C. L., Hieger, I., Kennaway, E. L., and Mayneord, W. V.: Chemical compounds as carcinogenic agents. Am. J. Cancer, 29: 219–259 (1937).

(*2*) Cook, J. W., and Kennaway, E. L.: Chemical compounds as carcinogenic agents. First supplementary report; literature of 1937. Am. J. Cancer, 33: 50–97 (1938).

(*3*) Cook, J. W., and Kennaway, E. L.: Chemical compounds as carcinogenic agents: Second supplementary report; literature of 1938 and 1939. Am. J. Cancer, 39: 381–428 and 521–582 (1940).

(*4*) Fieser, L. F.: Carcinogenic activity, structure and chemical reactivity of polynuclear aromatic hydrocarbons. Am. J. Cancer, 34: 37–124 (1938).

(*5*) Shimkin, M. B.: Biologic testing of carcinogens. I. Subcutaneous injection technique. J. Nat. Cancer Inst., 1: 211–223 (1940).

(*6*) Trevan, J. W.: The error of determination of toxicity. Proc. Roy. Soc., London, s. B, 101: 483–514 (1927).

(*7*) Gaddum, J. H.: III. Methods ot biological assay depending on the quantal response. M. Research Council, Spec. Rep. Ser. No. 183. H. M. Stationery Office, London (1933).

(*8*) Bliss, C. I.: The calculation ot the dosage—mortality curve. Ann. Appl. Biol., 22: 134–167 (1935).

(*9*) Burn, J. H.: Biological standardization. Oxford Univ. Press, New York and London (1937).

(*10*) Foster, R. H. K.: Standardization of safety margin. J. Pharmacol. & Exper. Therap., 65: 1–17 (1939).

(*11*) Bourdillion, R. B., Bruce, H. M., Fischman, C., and Webster, T. A.: The quantitative estimation of Vitamin D by radiography. M. Research Council Spec. Rep. Ser. No. 158. H. M. Stationery Office, London (1931).

(*12*) Irwin, J. O.: Statistical method applied to biological assays. Suppl. to J. Roy. Statis. Soc., 4: 1–60 (1937).

(*13*) Hemmingsen, A. M.: The accuracy of insulin asssay on white mice. I–II. Quart. J. Pharm. & Pharmacol., 6: 39–80, 187–218 (1933).

(*14*) Emmens, C. W.: Variables affecting the estimation of androgenic and oestrogenic activity. M. Research Council Spec. Rep. Ser. No. 234. H. M. Stationery Office, London (1939).

(*14a*) ———: The dose/response relation for certain principles ot the pituitary gland, and ot the serum and urine ot pregnancy. J. Endocrinol., 2: 194–225 (1940).

(*15*) Shimkin, M. B., and Andervont, H. B.: Effect of foster nursing on the response of mice to estrogens. J. Nat. Cancer Inst., 1: 599–605 (1941).

(*16*) Bryan, W. R., and Beard, J. W.: Estimation of purified papilloma virus protein by infectivity measurements. J. Infect. Dis., 65: 306–321 (1939).

(*17*) Bryan, W. R., and Beard, J. W.: Host influence in the characterization of response to the papilloma protein and to vaccinia virus. J. Infect. Dis., 67: 5–24 (1940).

(*18*) Bryan, W. R., and Beard J. W.: Correlation of frequency of positive inoculations with incubation period and concentration of purified papilloma protein. J. Infect. Dis., 66: 245–253 (1940).

(*19*) Bryan, W. R., and Beard, J. W.: Quantitative studies on the neutralization of purified papilloma virus. I. The relation between serum, total virus and free virus. J. Infect. Dis., 68: 133–170 (1941).

(*20*) Bryan, W. R., and Beard, J. W.: Studies on the purification and properties of the rabbit-papilloma—virus protein. J. Nat. Cancer Inst., 1: 607–673 (1941)

(*21*) Bliss, C. I.: The comparison of dosage—mortality data. Ann. Appl. Biol., 22: 307–333 (1935).

(*22*) ———: The determination of the dosage-mortality curve from small numbers. Quart. J. Pharm. & Pharmacol., 11: 192–216 (1938).

(*23*) Andervont, H. B., and Shimkin, M. B.: Biologic testing of carcinogens. II. Pulmonary-tumor-induction technique. J. Nat. Cancer Inst., 1: 225–239 (1940).

(*24*) Shimkin, M. B.: Length of survival of mice with induced subcutaneous sarcomas. J. Nat. Cancer Inst., 1: 761–765 (1941).

(*25*) Pearson, K.: Tables for statisticians and biometricians. Univ. Press, Cambridge (1924).

(*26*) Fisher, R. A., and Yates, F.: Statistical tables for biological, agricultural and medical research. Oliver & Boyd, London (1938).

(*27*) Dobrovolskaia-Zavadskaia, N.: Les doses minimes de 1, 2, 5, 6-dibenzanthracène capables de produire, en une seule injection sous-cutanée un cancer chez la souris. Compt. rend. Soc. de biol., 129: 1055–1057 (1938).

(*28*) Lettinga, T. W.: De carcinogene wurking van kleine doses 1, 2, 5, 6-dibenzanthraceen. Academisch Proefschrift, Amsterdam, Van Gorcum & Co., Assen (1937).

(*29*) Shimkin, M. B., and Andervont, H. B.: Comparative carcinogenicity of three carcinogenic hydrocarbons. J. Nat. Cancer Inst., 1: 57–62 (1940).

(*30*) Snedecor, G. W.: Calculation and interpretation of analysis of variance and covariance. Collegiate Press, Inc., Ames, Iowa (1934).

(*31*) Sall, R. D., and Shear, M. J.: Studies in carcinogenesis. XII. Effect of basic fraction of creosote oil on the production of tumors in mice by chemical carcinogens. J. Nat. Cancer Inst., 1: 45–55 (1940).

(*32*) BONSER, G. M., and ORR, J. W.: Morphology of 160 tumours induced by carcinogenic hydrocarbons in subcutaneous tissue of mice. J. Path. & Bact., 49: 171–183 (1939).

(*33*) DUNNING, W. F., CURTIS, M. R., and BULLOCK, F. D.: The respective roles of heredity and somatic mutation in the origin of malignancy. Am. J. Cancer, 28: 681–712 (1936).

(*34*) DUNNING, W. F., CURTIS, M. R., and WOOD, F. C.: Volume of injection and concentration of the carcinogenic chemical as factors in the initiation of the malignant process and their bearing on the somatic mutation hypothesis. Am. J. Cancer, 39: 70–93 (1940).

(*35*) IBALL, J.: The relative potency of carcinogenic compounds. Am. J. Cancer, 35: 188–190 (1939).

The *d*-Peptidase Activity of Serum as an Alleged Diagnostic Test for Cancer

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In 1939 Waldschmidt-Leitz and Mayer (*1*) published a clinical test for cancer based on their experiments which apparently showed that the serum of patients with carcinoma always hydrolyzed over 50 percent of the racemic peptides, *dl*-leucylglycine, *dl*-glutamylglycine, and *dl*-glutamylglycine ethyl ester, indicating the ability to hydrolyze the optically unnatural isomer of the racemic peptide. This followed the report of Kögl and Erxleben (*2*) that racemic mixtures of amino acids, especially glutamic acid, could be isolated from neoplastic and not from normal tissues.

Both of these reports have stimulated considerable research in the fields of protein chemistry and of proteolytic enzymes. While most investigators (*3–8*) have been unable to confirm the work of Kögl and Erxleben, these authors continue to reaffirm their original research (*9–11*). Johnson (*12*) and Arnow and Upsahl (*13*) have shown that racemic glutamic acid was formed by acid hydrolysis of normal *l*-glutamic acid and that the racemic glutamic acid isolated from tissues may be an artifact.

The attempts to prove the presence of *d*-peptidase activity in the blood serum of patients with carcinoma have met with varying results. Bayerle and Podloucky (*14–15*) found that the blood serums from 3 out of 50 patients with carcinoma digested more than 50 percent of *dl*-leucylglycine. The serums from 4 patients with carcinoma and from 3 rabbits with Brown-Pearce tumor did not hydrolyze *d*-leucylglycine. The serums from 3 patients with carcinoma did not hydrolyze *d*-leucylglycylglycine. These authors state that they found an increased splitting of *dl*-leucylglycine by the serums of patients with carcinoma over that of normal serums, but it rarely reached more than 50 percent. Extracts of Brown-Pearce tumor also did not hydrolyze *d*-leucylglycine.

Euler and Skarżyński (*16–17*) reported results which showed that the hydrolysis of the unnatural *d*-peptide took place more slowly than that of the *l*-peptide, and that both the normal serums and the serums of patients with carcinoma digested the *d*-form if the incubation period was prolonged (124–144 hours). Eight of ten serums from patients with carcinoma, and 8 of 10 normal serums hydrolyzed *d*-leucylglycine after 72 hours, and all of the serums had digested the *d*-form after 144 hours. They also found that the serum of one patient with carcinoma, as well as the serums of normal rats and of rats bearing Jensen sarcoma hydrolyzed the *d*-glutamic acid peptide of Bruckner and Ivanovics (*18*). The serum of rats with Jensen sarcoma did not hydrolyze *d*-leucylglycine, or more than 50 percent of the *dl*-alanylglycine. Glycerine extracts of Jensen sarcoma tissue also did not digest over 50 percent of *dl*-alanylglycine.

Abdĕrhalden and Caesar (*19*) found that extracts of human hepatic carcinoma, normal rabbit liver and kidney digested glycyl-*d*-norleucine and glycyl-*d*-isoleucine. These extracts did not digest over 50 per-

cent of the racemic compound, *dl*-leucylglycine. Glycyl-*d*-norleucine and glycyl-*d*-isoleucine were not hydrolyzed by the serums of 3 patients with carcinoma. More recently Abderhalden and Abderhalden (*20*) have reported that, of 41 patients with carcinoma, the serums of 9 digested both *d*-leucylglycylglycine and *d*-alanylglycine. Glycyl-*d*-leucine and *d*-alanyl-*l*-tryptophane were not hydrolyzed by any of these serums. Of 49 patients without cancer, the serum of only one (a patient with hyperthyroidism) digested *d*-leucylglycylglycine and *d*-alanylglycylglycine. The patients whose serums showed *d*-peptidase activity excreted *d*-peptidase in the urine.

Berger and Johnson (*21–22*) have described a method for concentrating leucylpeptidase from different sources and have shown that it is activated by Mn^{++}, Mg^{++}, and cysteine if used in proper concentration. They expressed the opinion that the leucylpeptidases do not act specifically upon one optical form of the substrate, but that one enzyme acts on both the *d*- and *l*-leucylpeptides at different rates. They found that many common proteolytic systems were capable of hydrolyzing the unnatural peptide, *d*-leucylglycine, in the presence of an activator, such as Mn^{++}+cysteine. They reported (*23*) that the serums of three patients with gastric carcinoma did not hydrolyze *d*-leucylglycine with or without activation with Mn^{++}. Serums of normal rats as well as serums of rats with Flexner-Jobling carcinoma digested *d*-leucylglycine extremely slowly. With the concentrated extract of the Flexner-Jobling carcinoma digestion of *d*-leucylglycine occurred when Mn^{++} was used as activator.

In the present study we have tested the *d*-peptidase activities of the serums of patients with carcinoma and of control serums of patients suffering from diseases other than carcinoma, and of normal persons. Similar studies were made on the peptidase activities of the serum of normal rats and of those bearing Jensen sarcoma. Moreover, since Waldschmidt-Leitz and Mayer have suggested that the *d*-peptidases may have diffused into the blood from the tumor tissue, we have compared the peptidase activities of normal and of neoplastic tissues.

EXPERIMENTAL METHODS

Source of Enzymes

The human blood [1] was drawn from 8 patients whose diagnosis of carcinoma was based upon histological examination of biopsy specimens,[2] and from 11 other patients whose diagnosis of carcinoma was based on clinical or radiological evidence.

The Jensen rat sarcomas had been transplanted into the muscles of the hind legs of Buffalo strain rats and were allowed to grow for 21 days, or until they had reached maximum size without ulceration. The Brown-Pearce testicular tumors were allowed to grow for 30 days to obtain a maximum amount of growing tissue. The malignant hepatoma (No. 16) [3] was a subcutaneous transplant from a primary hepatoma which developed in a rat after feeding *p*-dimethylaminoazobenzene for 7½ months.

The Digests

The serums of human blood samples and the pooled serums of rat blood were separated by centrifuging from the clotted blood after standing at 10° C. overnight. The samples were kept on ice before and

[1] *We are indebted to Dr. Lynn F. Cooper and Dr. Ben F. Jones, of the staff of the Washington Home for Incurables, and to Dr. Bocock, Dr. Veal, and Dr. Parks, of Gallinger Municipal Hospital, for the blood samples.*

[2] *Histological examination of the biopsy specimens, was made by Dr. F. Eberson, director of the laboratory, Gallinger Municipal Hospital.*

[3] *The hepatoma tissue was kindly furnished by Dr. Julius White, of this Institute.*

after separation of the serum. Most of the experiments with blood serum followed exactly the technique of Waldschmidt-Leitz and Mayer in which 3 parts of serum were added to 2 parts of N-ammonium hydroxide-ammonium chloride buffer. To each cubic centimeter of these digests was added 14.96 mg. of *d*- or *dl*-leucylglycine. The pH was adjusted to 8.0 electrometrically, and the incubation temperature was 30°. Other digestions were made with phosphate buffer at 38°. *d*-Leucylglycylglycine and *dl*-glutamylglycine were used in a concentration of 0.05 millimoles per cubic centimeter.

The tissue and serum peptidases were prepared according to the method described by Berger and Johnson (*21*). The tissues were ground in a mortar and extracted with from four to five times their weight of 20 percent by volume aqueous glycerol, without pH adjustment. After extraction for several hours at 10° C., the extracts were adjusted to pH 6.0 and treated with an equal volume of acetone. The precipitate was separated by centrifuging and taken up in a volume of water at pH 6.8 equal to the weight of the original sample of tissue. The serums were treated in the same manner by adding the aqueous glycerol directly to the serum.

The digests were made by adding 1 cc. of peptidase solution to 3 cc. of phosphate buffer containing the substrates which were used in a concentration of 0.05 millimoles per cubic centimeter. The pH of the *d*- and *dl*-leucylglycine and *dl*-glutamylglycine digests was 8.0; of the *d*-leucylglycylglycine digest, 7.5.

The degree of hydrolysis was determined by alkalimetric titration in alcohol using thymolphthalein as indicator acacording to Grassmann and Heyde (*24*). The percentage hydrolyses are given in the tables for splitting of one peptide bond of the racemic mixture. Titrations were made daily for 5 days following the procedure of Waldschmidt-Leitz and Mayer. The titration value obtained without the addition of a substrate was subtracted in each case. The synthetic substrates were found to be stable under the conditions of the experiment.

In some digests M/200 or M/3000 cysteine and M/1000 Mn^{++} were used as activators.

RESULTS

The serum of 1 of 19 patients with carcinoma was able to digest more than 50 percent of the *dl*-leucylglycine upon repeated testing (table 1). This serum also digested 37 percent of *d*-leucylglycine in 48 hours. This patient, M. S., with an adenocarcinoma of the breast and metastases to the abdomen and bones, as shown by radiological examination, probably had the most extensive carcinomatous growths in the series of patients. The serum of one other patient with carcinoma hydrolyzed 79 percent of the *dl*-leucylglycine, but this degree of digestion was not repeated with a blood serum taken 1 month later. The digestion of the two racemic dipeptides to 50–55 percent by some of the serums from the patients with carcinoma probably signifies that all of the peptide containing the natural amino acid, *l*-leucine or *l*-glutamic acid, was hydrolyzed. There was no evidence that X-ray treatment affected the peptidase activity of the serums.

The digestion of *dl*-leucylglycine by the serum of patients with diseases other than carcinoma, and by the serum of normal persons was less than 50 percent in all cases .except one, A. In this case the value of 58 percent hydrolysis fell to 13 percent when this patient's blood was tested 1 month later (table 2). The highest value obtained with the normal serums was 39 percent hydrolysis.

TABLE 1.—*The hydrolysis of* dl-*leucylglycine and* dl-*glutamylglycine by the blood serums of patients with carcinoma*

[The maximum percentage of hydrolysis obtained and the digestion period in hours required for this maximum hydrolysis are given]

Experiment No.	Sex and age of patient	Diagnosis [1]	Hydrolysis			
			dl-Leucylglycine		*dl*-Glutamylglycine	
			Pct.	*Hrs.*	*Pct.*	*Hrs.*
1	F. H. male, 60 yrs	Adenocarcinoma, sigmoid	50	96	45	120
2	N. F. female, 45 yrs	Squamous-cell carcinoma, cervix	28	96	40	96
3	H. P. male, 63 yrs	Squamous-cell carcinoma, ear	48	120	38	120
4	S. L. female, 31 yrs	Carcinoma, bladder	37	120	45	72
5	M. B. female, 65 yrs	Adenocarcinoma, rectum	30	72	33	120
6	K. G. male, 57 yrs	Metastatic adenocarcinoma in mesenteric lymph node (primary, stomach?).	40	120	45	96
7	L. A. female, 37 yrs	Squamous-cell carcinoma of cervix	54	120	55	48
8	D. male, 67 yrs	Carcinoma, prostate	32	96	.....	.....
9	K. female, 70 yrs	Carcinoma of colon (colostomy performed)	79	120	.....	.....
	One month later		48	96	.....	.....
10	M. S. female, 60 yrs	Adenocarcinoma, breast, with metastases to abdomen and bones.	88	120	.....	.....
	One month later		79	120	.....	.....
11	M. female, 58 yrs	Adenocarcinoma, breast	55	120	.....	.....
12	M. O. female, 77 yrs	do	29	72	.....	.....
13	T. male, 82 yrs	Carcinoma, nose	31	120	.....	.....
14	M. male, 64 yrs	Carcinoma, recto-sigmoid	31	120	.....	.....
15	R. male, 64 yrs	Carcinoma, lung with metastases	53	120	.....	.....
16	P. male, 63 yrs	Carcinoma, colon	39	96	.....	.....
17	M. M. female, 70 yrs	Carcinoma, breast	44	120	25	72
18	M. R. female, 44 yrs	Carcinoma, bladder	35	120	20	120
19	A. B. female, 65 yrs	Carcinoma, colon	22	120	25	96

[1] The diagnoses of the first eight patients were based on examination of biopsy specimens. The diagnoses of the other patients were based on clinical or radiological evidence.

TABLE 2.—*The hydrolysis of* dl-*leucylglycine by blood serums of patients without carcinoma and of normal persons*

[The maximum percentage hydrolysis obtained and the digestion period in hours required for this maximum hydrolysis are given]

Sex and age of patient	Diagnosis	Hydrolysis of *dl*-leucylglycine	
		Percent	*Hours*
L. male, 65 yrs	Arteriosclerosis	31	96
McG. male, 77 yrs	Hypertension	29	120
A. female, 72 yrs	Myxodema, ascites	58	120
Same, 1 month later		13	120
W. female, 83 yrs	Hypertrophic arthritis, hypertension.	37	96
C. female, 79 yrs	Congestive heart failure.	18	48
Same, M/200 cysteine added.		15	48
Normal:			
1. Female, 48 yrs		37	120
Same, M/200 cysteine added.		36	72
2. Male, 47 yrs		25	120
3. Male, 40 yrs		18	96
4. Male, 35 yrs		32	120
5. Female, 33 yrs		25	120
6. Male, 25 yrs		0	.....
7. Male, 25 yrs		39	24
8. Male, 20 yrs		17	120

The results shown in tables 3 and 4 indicate that the serums of normal rats hydrolyzed *dl*-leucylglycine more extensively than the serums of rats bearing Jensen sarcoma. Without activation most of the lots of pooled serum from normal animals digested more than 50 percent of the *dl*-leucylglycine, while with cysteine three lots of normal serums were activated to digest considerably more than 50 percent. One lot of serums from the rats bearing tumors was activated to digest 56 percent. The serums from the normal rats also showed more extensive digestion of the *d*-leucylglycine than the serums of the tumor-bearing rats.

There is some indication that the serums from the older, heavier, normal rats hydrolyzed the *dl*-leucylglycine more than did the serums of the younger normal rats. The rats bearing the Jensen sarcomas weighed from 150 to 250 gm. However, the lower weight of the sarcoma-bearing rats may not entirely explain the difference in activity since the serums of these animals could not be activated to digest much more than 50 percent of the *dl*-leucylglycine, while the serums of the younger normal rats in this lower weight range could be activated to digest considerably

TABLE 3.—*The hydrolysis of* dl- *and* d-*leucylglycine,* d-*leucylglycylglycine, and* dl-*glutamylglycine by the pooled serum of rats bearing Jensen sarcoma* [1]

[The maximum percentage of hydrolysis obtained and the digestion period in hours required for this maximum hydrolysis are given]

Experiment No.	Hydrolysis									
	dl-Leucylglycine				*d*-Leucylglycine		*d*-Leucylglycyl-glycine		*dl*-Glutamylglycine	
	No activator		With activator		No activator		No activator		No activator	
	Percent	*Hours*	*Percent*	*Hours*	*Percent*	*Hours*	*Percent*	*Hours*	*Percent*	*Hours*
1	66	120								
2	48	24	38	72						
3	31	72	42	24						
4	10	24	56	96						
5	58	96	46	72	11	72				
6	29	24			16	24				
7	51	48					0		46	48
8	39	120					0		56	120
9	51	24	52	72					58	48

[1] In experiments 5, 6, 8, and 9, phosphate buffer was used and the digest was incubated at 40° C. In all other digests NH_4OH–NH_4Cl buffer was used and the temperature was 30° C. M/3000 Mn was used in experiment 2, and M/3000 cysteine plus M/1000 Mn was used in experiment 5 in attempts to activate the enzyme. M/157.5 cysteine was used to activate in experiments 3, 4, and 9. In experiment 5 the serum did not digest *d*-leucylglycine without an activator.

more than 50 percent of this racemic peptide in most cases.

Dietary experiments have been reported which demonstrate the differences in the ability of young and of adult rats to utilize *d*-leucine. Rose (*25*) found that the young, growing rats could not utilize *d*-leucine when fed a leucine-deficient diet, while Ratner, Schoenheimer, and Rittenberg (*26*) report that *d*-leucine was deaminized and used for tissue proteins by adult rats on a complete diet.

The sex of the rats did not seem to influence the degree of peptidase activity of the serum.

The *dl*-glutamylglycine was digested

TABLE 4.—*The hydrolysis of* dl- *and* d-*leucylglycine,* d-*leucylglycylglycine, and* dl-*glutamylglycine by the pooled serum of normal rats* [1]

[The maximum percentage of hydrolysis and the digestion period in hours required for this maximum hydrolysis are given]

Experiment No.	Average weight	Sex	Hydrolysis									
			dl-Leucylglycine				*d*-Leucylglycine		*d*-Leucylglycyl-glycine		*dl*-Glutamyl-glycine	
			No activator		With activator		No activator		No activator		No activator	
	Grams		*Percent*	*Hours*	*Percent*	*Hours*	*Percent*	*Hours*	*Percent*	*Hours*	*Percent*	*Hours*
1	150	M	61	120			32	120				
2	130	M	77	120								
3	165	M+F	31	48	71	24						
4	165	F	43	72	69	48						
5	250	F	81	72	84	48			27	48	56	48
6	280	F	41	120					0		36	96
7	325	M	90	120			73	120				
8	325	M	71	72			51	72				
9	325	F	49	24	103	120						
10	400	M	67	72							54	72
11	435	M	62	24					31	48	53	24
12	450	M+F	73	48								
13	450	M+F	98	120								
14	450	M+F	94	48								
15	450	M+F	87	48	74	48						

[1] In experiments 2, 3, 8, and 13, phosphate buffer was used and the digest was incubated at 40° C. In all other experiments NH_4OH–NH_4Cl buffer was used and the temperature was 30° C. In experiments 3, 4, 5, and 9, M/200 cysteine was used to activate. M/1000 Mn^{++} was used to activate in experiment 15.

TABLE 5.—*The hydrolysis of* dl-*leucylglycine,* d-*leucylglycylglycine, and* dl-*glutamylglycine, by the purified peptidases prepared from the serums of normal and of Jensen sarcoma-bearing rats*

[The maximum percentage of hydrolysis obtained and the digestion period in hours required for this maximum hydrolysis are given]

Source of enzyme	Hydrolysis											
	dl-Leucylglycine				*d*-Leucylglycylglycine				*dl*-Glutamylglycine			
	No cysteine		With cysteine [1]		No cysteine		With cysteine		No cysteine		With cysteine	
	Percent	*Hours*	*Percent*	*Hours*	*Percent*	*Hours*	*Percent*	*Hours*	*Percent*	*Hours*	*Percent*	*Hours*
Serums of rats bearing Jensen sarcoma	44	72	50	72	.....	.....	.....	.....	25	24	21	24
	72	72	.....	.....	19	24	.....	.....	27	120	.....	.....
	83	24	.....	.....	.....	.....	.....	.....	29	72	.....	.....
	63	48	61	120	0	.....	18	96	24	48	45	4
Serums of normal rats	64	120	51	72	.....	.....	.....	.....	24	120	.....	.....
	58	120	53	24	37	120	18	48	39	120	21	120
	42	72	.....	.....	8	48	.....	.....	6	24	.....	.....
	39	72	49	72	25	120	.....	.....	18	72	.....	.....

[1] M/200 Cysteine was used.

equally well by the serums of normal and of tumor-bearing rats.

When the peptidases of the two types of serum were purified according to the method of Berger and Johnson, the preparations from the serums of the sarcoma-bearing rats were more active than the preparations from the normal serums (table 5). Two lots of serum from tumor-bearing rats digested much more than 50 percent of *dl*-leucylglycine after this treatment. Conversely, two normal serums were less active after this treatment. Further study of the activators or inhibitors of peptidase activity which are present in serum might explain these results.

The peptidases prepared from tumor tissues digested much more *dl*-leucylglycine than did those prepared from normal livers, although there is no definite evidence of *d*-peptidase activity (table 6). A comparison between the peptidase activities of a tumor tissue and its tissue of origin is possible in the case of the transplanted hepatoma and the normal liver of the rat. Here it is seen that the peptidase activity of the malignant tissue is more active than that from the normal liver.

TABLE 6.—*The hydrolysis of* dl-*leucylglycine and* dl-*glutamylglycine by purified peptidase prepared from normal and neoplastic tissues*

[The maximum percentage of hydrolysis obtained and the digestion period in hours required for this maximum hydrolysis are given]

Source of enzyme	Hydrolysis			
	dl-Leucylglycine		*dl*-Glutamylglycine	
	Per cent	*Hours*	*Per-cent*	*Hours*
Liver of normal rat	15	2	.....	.....
	25	2	.....	.....
Liver of rat with transplanted hepatoma produced by feeding *p*-dimethylaminoazobenzene	16	48	40	96
Liver of rabbit with Brown-Pearce testicular tumor	29	120	31	72
Jensen rat sarcoma	45	2	.....	.....
	49	2	.....	.....
Transplanted hepatoma produced originally by feeding *p*-dimethylaminoazobenzene	46	20	51	120
Brown-Pearce testicular tumor	59	120	48	120

DISCUSSION

From the varying results reported here and elsewhere on the peptidase activities of human and animal serums, it is seen that the serum peptidase lacks specificity for *l*-leucylglycine which contains the naturally occurring *l*-leucine. A tabulation of the published results with this test shows that the serums of 28 of 129 patients with carcinoma reacted positively by digesting a *d*-peptide, while the serums of

13 of a total of 115 serums from patients suffering from diseases other than cancer and from normal persons also gave positive tests. It is also seen that the rate of hydrolysis of the rat serum leucylpeptidase may be controlled by the presence of activators. This is in agreement with the results of Berger and Johnson (*22*) who have shown that a metal or reducing agent alone may cause a substantial activation of the hydrolysis of *dl*-leucylglycine, but a combination of the two is far superior. Mn^{++} or Fe^{++} alone or combined with a reducing agent such as cysteine or glutathione activated the dipeptidase activity of yeast, hog intestinal mucosa, and of Flexner-Jobling rat carcinoma tissue. They found that peptidases from chick mucosa, yeast autolysate, and malt were able to split *d*-leucylglycine about one-thirtieth as rapidly as *dl*-leucylglycine in the presence of suitable activators such as Mn^{++} or Mn^{++}+cysteine. From these activation studies on leucylpeptidase, it would seem that fluctuations in the normal reducing substances of the blood serums might account for the variation in the *d*-leucylpeptidase activity of both the normal serums and the serums of patients with carcinoma.

Because an enzymatic diagnostic test for cancer would be very valuable, it is worthwhile to consider the relationship of the enzymes of the serum to the spatial configuration of the amino acids in the proteins of the tumor tissue. The presence of enzymes in the organs or blood capable of hydrolyzing *d*-peptides does not necessarily signify the presence of *d*-amino acids in the tissue proteins since many *d*-amino acids have been shown to be inverted by normal enzyme systems, first by deamination (*27*) and then by amination processes, to amino acids of the *l*-series before synthesis into tissue proteins. As an illustration of this inversion, Conrad and Berg (*28*) fed rats on a histidine-deficient diet which had been supplemented with *d*-histidine and found only *l*-histidine in the tissues. And recently Ratner, Schoenheimer, and Rittenberg (*26*), using isotopes, have followed the inversion of a *d*-amino acid more closely by feeding *d*-leucine which contained deuterium as a marker for the carbon chain and N^{15} in the amino groups. Only the *l*-form of leucine containing a considerable amount of deuterium and a very low proportion of N^{15} was isolated from the proteins of the liver and the rest of the body.

Our recently published work (*29–30*) has shown that the purified cathepsins which are responsible for the synthesis and autolysis of the proteins of rat liver, kidney, spleen, and Jensen sarcoma did not hydrolyze peptides formed from *d*-leucine and *d*-glutamic acid. It was also demonstrated that the purified cathepsins of normal liver are able to digest the proteins and peptides of tumor tissue, indicating that the growth of the tumor was not due to the inability of the enzymes of normal tissue to digest the tumor peptides containing *d*-amino acids, as suggested by Kögl and Erxleben.

SUMMARY

The studies reported herein show that the *d*-peptidase activity of serum cannot be regarded as a test for cancer since the serum of only 1 of 19 patients with carcinoma reacted positively, i. e., digested more than 50 percent of the racemic dipeptide, *dl*-leucylglycine. None of the serums of patients suffering from diseases other than carcinoma or of normal persons digested more than 50 percent of *l*-leucylglycine.

The serums of old normal rats in many cases digested much more than 50 percent of *dl*-leucylglycine and also digested *d*-leucylglycine, indicating a lack of antip-

odal specificity for the naturally occurring optical isomer of the peptide. The hydrolysis of *dl*-leucylglycine by normal rat serum could be activated by the addition of cysteine. The serum of rats bearing Jensen sarcomas seldom digested more than 50 percent of the *dl*-leucylglycine. However, when the peptidase fraction of the serum was prepared, the hydrolysis increased to more than 50 percent in some instances. The hydrolysis of *dl*-glutamylglycine by either normal rat serum or the serum of rats bearing Jensen sarcoma seldom exceeded 50 percent.

The peptidase preparations from tumor tissues, including Jensen rat sarcoma, a transplanted hepatoma produced originally by feeding rats *p*-dimethylaminoazobenzene, and the Brown-Pearce testicular tumor, showed more ability to hydrolyze the *dl*-leucylglycine than the peptidase preparations from normal livers. The maximum digestion of 59 percent by the Brown-Pearce tumor peptidase would seem to indicate that these tumor-tissue peptidases possess little, if any, *d*-peptidase activity.

The relation of possible *d*-peptidases in the blood serum to the configuration of the amino acids in the tissue proteins is discussed.

The technical assistance of Richard Holbrook is acknowledged.

REFERENCES

(*1*) Waldschmidt-Leitz, E., and Mayer, K: Über sterische Auslese durch Peptidasen in normalen und carcinomatösen Seren. Ztschr. f. physiol. Chem., 262: IV–VI (1939).

(*2*) Kögl, F., and Erxleben, H.: Zur Ätiologie der malignen Tumoren. 1. Mitteilung über die Chemie der Tumoren. Ztschr. f. physiol. Chem., 258: 57–95 (1939).

(*3*) Chibnall, A. C., Rees, M. W., Tristam, G. R., Williams, E. F., and Boyland, E.: The glutamic acid of tumor proteins. Nature, London, 144: 71–72 (1939).

(*4*) Chibnall, A. C., Rees, M. W., Williams, E. F., and Boyland, E.: The glutamic acid of normal and malignant tissue proteins. Biochem. J., 34: 285 (1940).

(*5*) Chibnall, A. C., Rees, M. W., Williams, E. F., and Boyland, E.: Glutamic acid of proteins. Nature, London, 145: 311–312 (1940).

(*6*) Graff, S.; Glutamic acid in malignant tumors. J. Biol. Chem., 130: 13–17 (1939).

(*7*) Johnson, J. M.: A comparison of the optical forms of glutamic acid from normal and cancerous tissue. J. Biol. Chem., 132: 781 (1940).

(*8*) ———: Glutamic acid from normal and cancerous tissue. J. Nat. Cancer Inst., 1: 113–117 (1940).

(*9*) Kögl, F., Erxleben, H., and Akkerman, A. M.: Isolierung der Glutaminsäure aus Tumorproteinen. 2. Mitteilung über die Chemie der Tumoren. Ztschr. f. physiol. Chem., 261: 141–153 (1939).

(*10*) Kögl, F., and Erxleben, H.: Zur Stereochemie der Proteine von Myomen und anderen Geschwülsten. 3. Mitteilung über die Chemie der Tumoren. Ztschr. f. physiol. Chem., 261: 154 (1939).

(*11*) Kögl, F., Erxleben, H., and Herken, H.: Weitere Beiträge zur Stereochemischen Analyse von Tumorproteinen. 4. Mitteilung über der Chemie der Tumoren. Ztschr. f. physiol. Chem., 263: 107–124 (1940).

(*12*) Johnson, J. M.: The racemization of glutamic acid. J. Biol. Chem., 134: 459 (1940).

(*13*) Arnow, L. E., and Opsahl, J. C.: Partial racemization of glutamic acid in boiling hydrochloric acid solutions. J. Biol. Chem., 133: 765–766 (1940).

(*14*) Bayerle, H., and Podloucky, F. H.: Zur Frage des Vorkommens von *d*-Peptidasen in normalen und carcinomatösen Organ. Biochem. Ztschr., 304: 259–265 (1940).

(*15*) Bayerle, H., and Podloucky, F. H.: Zur Frage des Vorkommens von sterisch auslesenden Enzymen in carcinomatösen Organismus. Feststellung zu zwei vorläufigen Mitteilungen von E. Waldschmidt-Leitz und Mitarbeitern. Ztschr. f. physiol. Chem., 264: 189–195 (1940).

(*16*) v. Euler H., and Skarżyński, B.: Zur Kenntniss der Spaltung optisch aktiver Dipeptide durch maligne Tumoren und durch serum. I–II. Arkiv for Kemi, Mineral. och Geol. 14 B, Nos. 2–3 (1940).

(*17*) v. EULER H., and SKARŻYŃSKI, B.: Über die Spaltung von Dipeptiden in carcinomatösen und nicht carcinomatösen Seren. Ztschr. f. physiol. Chem., 265: 133–146 (1940).

(*18*) BRUCKNER, V, and IVANOVICS, G.: Über das natürliche Vorkommen und über eine einfache biologische Gewinnungsart der *l* (−)-Glutaminsäure. Ztschr. f. physiol. Chem., 247: 281–284 (1937).

(*19*) ABDERHALDEN, E., and CAESAR, G.: Über das Verhalten von Dipeptiden, in denen Aminosäuren der *d*-Reihe enthalten sind, bei der Einwirkung von Pressäften aus Geweben und von Blutserum. Fermentforschung, 16: 299–308 (1940).

(*20*) ABDERHALDEN, E., and ABDERHALDEN, R.: Beitrag zum Problem des Vorkommens von Polypeptidasen im Blutserum und im Harn von Carcinomträgern, die Polypeptide zu spalten vermögen in denen *d*-Aminosäuren enthalten sind. Ztschr. f. physiol. Chem., 265: 253–265 (1940).

(*21*) BERGER, J., and JOHNSON, M. J.: The occurrence of leucylpeptidase. J. Biol. Chem., 133: 157–172 (1940).

(*22*) BERGER, J., and JOHNSON, M. J.: The activation of dipeptidases. J. Biol. Chem., 133: 639–640 (1940).

(*23*) BERGER, J., JOHNSON, M. J., and BAUMANN, C. A.: Enzymatic hydrolysis of *d*-peptides. J. Biol. Chem., 137: 389–395 (1941).

(*24*) GRASSMANN, W., and HEYDE, W.: Alkalimetrische Mikrobestimmung der Aminosäuren und Peptide. Ztschr. f. physiol. Chem., 183: 32–38 (1929).

(*25*) ROSE, W. C.: The nutritive significance of the amino acids and certain related compounds. Science, 86: 298–300, 1937).

(*26*) RATNER, S., SCHOENHEIMER, R., and RITTENBERG, D.: Studies in protein metabolism. XIII. The metabolism and inversion of *d* (+)—leucine studied with two isotopes. J. Biol. Chem., 134: 653–663 (1940).

(*27*) KREBS, H. A.: Untersuchungen über den Stoffwechsel der Aminosäuren im Tierkörper. Ztschr. f. physiol. Chem., 217: 191–227 (1933).

(*28*) CONRAD, R. M., and BERG, C. P.: The optical inversion of *d*-histidine in the animal body. J. Biol. Chem., 117: 351–363 (1937).

(*29*) Maver, M. E.: The separation of cathepsin from malignant and normal rat tissue. J. Biol. Chem. 131: 127–134 (1939).

(*30*) MAVER, M. E., JOHNSON, J. M., and THOMPSON, J. W.: The peptidase activities of the cathepsins of normal rat tissue and of Jensen rat sarcoma. J. Nat. Cancer Inst., 1: 675–686 (1941).

The Depolymerization of Thymonucleic Acid by an Enzyme System in Normal and Cancerous Hepatic and Mammary Tissues and in the Milk and Sera of Several Species

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INTRODUCTION

Historical and Critical Survey

The method of isolating thymonucleic acid from tissues has been to boil the minced tissues with strong alkali, followed by acidification, removal of the denatured proteins by filtration, and precipitation of the nucleic acid from the filtrate by addition of alcohol (*1*). This procedure leads to a granular product which dissolves in hot water. On cooling, the solution gels. Feulgen (*2*) found that a longer period of boiling the minced tissues with strong alkali resulted in obtaining a product which dissolved in hot water, but the solution failed to gel on cooling. He called the former preparation of thymonucleic acid the a-form, the latter the b-form. It seemed likely that the latter form was a degradation product of the former, and Feulgen himself admitted in a fairly recent publication the "schlechten Rufe" in which the b-form was regarded by many workers in the field. However, the a-form, which causes gelation of its aqueous solutions, was not above suspicion for it was inconceivable that a product isolated under such drastic conditions would be representative of conditions in the intact living tissue. Indeed, Hammarsten (*3*) in 1924 showed that the a-form of Feulgen was an artificial product of the action of strong alkali on "native" thymonucleic acid. The preparation of native nucleic acid in the highly polymerized form in which it presumably occurs in tissues was accomplished by Hammarsten by a process employing salt precipitation in the cold. The nucleic acid isolated by this method appears in the form of long white fibers which dissolve readily in cold water without gelling.

In spite of the purity and obvious advantages of the Hammarsten preparation of thymonucleic acid, very few workers outside of Sweden concerned themselves with it. It was ignored by Feulgen, and only in 1939 was a study published comparing the various preparations of thymonucleic acid. Schmidt, Pickels, and Levene (*4*) stated that the degree of polymerization of the preparations of thymonucleic acid by various workers decreased in the following order: Hammarsten, Neumann, Levene, Feulgen. In 1935 Feulgen (*2*), who was still concerned with the a- and b-forms of thymonucleic acid, announced the presence of an enzyme in pancreatin powder which transformed the a- into the b-form. According to Feulgen no phosphorus was thereby split from the nucleic acid. Feulgen named this enzyme "nucleogelase." Its action was indicated

by the transformation of the gel (a-form) to the watery consistence of the b-form. Schmidt, Pickels, and Levene (*4*) stated that the Hammarsten preparation of thymonucleic acid would not yield phosphorus by the action of intestinal phosphatase until it had been previously "depolymerized" by Feulgen's method with pancreatin.

The evidence offered by Feulgen and by Schmidt, Pickels, and Levene for the presence of a depolymerizing factor in pancreatin was indirect. A transformation of gel to sol, or the action of phosphatase is hardly evidence for depolymerization. There are salts and inert proteins in the commercial preparations of pancreatin. Greenstein and Jenrette (*5*) showed that pure proteins and a wide variety of salts have the property of depolymerizing nucleic acid. A small amount of a strongly active salt such as guanidine hydro-iodide immediately converted a gel of thymonucleic acid (Levene preparation) to the sol form. Greenstein and Jenrette (*5*) followed the extent of the depolymerization brought about by these agents by the diminution of the structural viscosity and the streaming birefringence of the thymonucleic acid. Again, when Schmidt, Pickels, and Levene (*4*) incubated the thymonucleic acid with pancreatin for 2 days at 37° C., no mention was made of the spontaneous depolymerization which the nucleic acid undergoes at an elevated temperature during a long period of time.

In addition to the omission of direct physical evidence for the depolymerization brought about by the nucleogelase enzyme of Feulgen, this investigator also failed to establish the necessary criteria for the presence of an enzyme. These consist, among other things, in the time course of the enzymic reaction, and in the use of inhibiting agents, more or less specific for enzymes, which affect the velocity of such reaction.

In view of the insufficient evidence for the presence of a depolymerizing enzyme described above, it was considered desirable to reinvestigate this problem, so important for a better understanding of the mechanisms involved in nuclear processes. In this communication is reported a study of the time course of the reaction of thymonucleic acid with extracts of normal and cancerous tissues. The study of lactating breast led to an extension of the investigation to the milk and the sera of several species. In order to assist in the interpretation of the results obtained, the earlier observations by the authors (*5*) on the depolymerization of nucleic acid by proteins, amino acids, and salts were extended to a consideration of the effect of these agents over a definite period of time.

EXPERIMENTAL PROCEDURE

Materials

The tissues investigated included the following pairs of tumors and normal control tissues: (1) Transplanted rat hepatic tumor No. 31 and normal and regenerating rat livers; (2) spontaneous mammary tumors in C3H mice and lactating mouse mammary tissues and hyperplastic mouse mammary tissues induced by stilbestrol. The transplanted hepatic tumor was in its fourth passage. Regenerating rat liver was obtained 2 days after operation. A description of these tissues, together with a discussion of the advantages and limitations of the study of tumor-normal tissue pairs, are given in a previous paper in this series by Greenstein, Jenrette, Mider, and White (*6*).

The tissues were ground in a mortar with a little sand and mixed with three times their weight of cold distilled water. After standing for 18 hours at 5° C., the

extracts were filtered through fine gauze and then centrifuged for 10 minutes at 1,500 r. p. m.

Some samples of milk were obtained fresh from various species of animals available in the laboratory. Samples of cow, mare, and goat milk were obtained raw. Human milk was donated by one of the local maternity hospitals. The sera of mice, rats, rabbits, and guinea pigs were obtained from blood drawn by cardiac puncture. Samples of human sera were obtained from the blood of several healthy adults of both sexes.

Three - times - crystallized egg albumin and horse-serum albumin were prepared by the usual procedures. Solutions of each protein were adjusted to pH 7.0 with dilute alkali.

Total nitrogen determinations were made on aliquots of each of the tissue extracts, protein solutions, and samples of sera and milk studied. Before the effects of these fluids were investigated on nucleic acid, they were adjusted with distilled water to definite and equal concentrations of total nitrogen in milligrams per cubic centimeter. By comparing the effects of these fluids at equal concentration of total nitrogen a certain uniformity in the experimental conditions is achieved. The total nitrogen figure for each solution includes that due to proteins, urea, amino acids, ammonium salts, etc. All of these components affect nucleic acid (*5*), and all vary in their absolute amounts and relative proportions in the fluids studied. To place each of these fluids on an equivalent total nitrogen basis is obviously a somewhat arbitrary procedure; nevertheless, it is difficult to conceive at the present time of a better method of placing the comparison of the fluids on a uniform basis. At least two and sometimes three different concentrations of total nitrogen were chosen. Several samples of each tissue were studied. The reproducibility of the results obtained were satisfactory throughout except in the single case of the lower concentrations of liver and hepatic-tumor extracts. No reason can be advanced at the present time for this discrepancy. Included in the present study is a comparison of the effect of the whole spontaneous mammary tumor in C3H mice and the mammary tumor in this strain from which the necrotic areas were carefully dissected.

The substrate upon which all the above agents act is a preparation of sodium thymonucleate prepared according to Hammarsten. A description of this particular preparation (No. 2) is given in the paper by Greenstein and Jenrette (*5*). Its aqueous solution has a pH of 6.9. Occasionally for convenience in the present paper, the thymonucleate substrate may be referred to as thymonucleic acid, or nucleic acid, but it is understood that it is always the completely neutralized tetrasodium salt of thymonucleic acid to which reference is made.

Method

The problem involves the extent of depolymerization of thymonucleic acid brought about by the interaction of various agents with the nucleic acid. How may this depolymerization be best observed over a period of time? Greenstein and Jenrette (*5*) showed that aqueous solutions of sodium thymonucleate possessed not only a very high viscosity, but that the value of the viscosity depended upon the pressure exerted in measuring it, e. g., solutions of the nucleate possess structural viscosity. In addition, the solutions of thymonucleate possess intense streaming birefringence. The action of any agent which causes a diminution in these properties may be interpreted on the basis that the nucleate molecules are being broken into smaller, less asymmetric

particles. This interpretation has been fully discussed by Greenstein and Jenrette (*5*), and in the analogous case of myosin by Edsall and Mehl (*7*). From the evidence of a repeating structure throughout the length of the thymonucleate chain (*8*) and from the evidence of organic chemistry (*1*), it appears legitimate to regard the disintegration of the asymmetric particles of thymonucleic acid as a depolymerization. The molecular weight of the component tetranucleotides is about 1,400, that of the nucleic acid about 1×10^6 (*9*). Approximately 800 tetranucleotides form the molecule of thymonucleic acid. The forces holding these components together are as yet unknown, but appear to be mainly of a saltlike nature (*5*). The partial or complete disintegration of so complex a structure might be expected to be reflected in considerable changes in its specific physical properties. The most sensitive physical properties of thymonucleate are its structural viscosity and streaming birefringence. We have, therefore, used changes in these properties as criteria for the disintegration of the thymonucleate molecule under the influence of the agents, and with the aid of some further evidence have assumed that the disintegration involves nothing more than a depolymerization. Quite independently, however, of any assumption as to the mechanisms involved, the use of these sensitive physical methods reveals the remarkable effects of many of the agents studied. On the other hand, the use of a method such as capillary viscosity imposes certain limitations on the physical nature of the agents used, for it is obvious that only homogeneous solutions may be employed.

We incubated at 30° C. mixtures of sodium thymonucleate with various tissue extracts, proteins, amino acids, and salt solutions, and samples of milk and of sera in the viscometer itself and measured the viscosity of the mixture at selected intervals. An external air pressure of 16.0-cm. H_2O was employed to force the solution through the capillary.[1] This pressure was selected because it occurs within the range where the viscosity of the thymonucleate is most sensitive to changes in its molecular shape. A brief description of the apparatus used has been given by the authors (*5*). Simultaneously, a portion of the mixture was examined from time to time for its streaming birefringence in the Edsall-Mehl apparatus (*7*).

The method of mixing is as follows: A 1-percent solution of sodium thymonucleate is prepared fresh before every determination. A 5-cc. portion of this solution is mixed with 5 cc. of the fluid whose effect is to be studied. Simultaneously, 5 cc. of the nucleate solution is mixed with an equal volume of distilled water. The former mixture is the test, the latter is the control. A further control is set up by diluting the fluid studied by an equal volume of distilled water. The partial volumes occupied by the nucleate and by the protein molecules are disregarded. The time of mixing is carefully noted, and selected volumes of the test and of the control mixtures are transferred to the viscometers. Readings of the relative viscosity of these mixtures are taken at various intervals. The viscosities are calculated as usual, relative to those of the extract, protein, or salt solutions, as the case may be. The concentration of sodium thymonucleate in test and in control is 0.5 percent. At 16-cm. H_2O pressure, the time required for the control to pass through the capillary used is about 9 minutes; the time similarly required for the diluted extract

[1] *The viscosity of a highly asymmetric molecule such as sodium thymonucleate has no meaning unless the external pressure is known.*

solution is about 14 seconds. A considerable time span is therefore provided, in which changes in the viscosity of the nucleate can be noted with ease and accuracy.

Determinations of inorganic phosphorus and ammonia were performed on an aliquot of the mixture of nucleic acid and extract, and on an aliquot of the extract at the beginning and at the end of the incubation period. It may be here stated that in no case, under the conditions used, was there any evidence of an analytically detectable change in the phosphorus or ammonia content of the mixture after incubation. There was, likewise, no apparent change in the pH. Thus, so far as one may determine at the present time, there are no chemical changes involved under the present conditions in the mixture of nucleate and fluids used. Any changes observed are apparently of a physical nature only. The failure to detect significant phosphatase activity in the mixtures used is similar to the findings of Maver and Voegtlin (*10*) who showed that thymonucleic acid is very little affected by tissue phosphatase within 4 hours at pH 7 and at 37° C. These investigators showed that tissue phosphatase has a major optimum activity at pH 8.8, and a minor optimum at an acid reaction. Our working conditions included a pH range of 6.8–7.2, an incubation period of 4 hours, and an incubation temperature of 30°. In view of the results of Maver and Voegtlin, our failure to observe any significant change in the phosphorus content is not unexpected.

The experimental results are given in the form of graphs, wherein the relative viscosities are plotted as a function of the time of incubation in the viscometers. The relative effect at equivalent nitrogen concentrations of each of the fluids studied can thus be visually compared. The order of reaction for the active preparations has not as yet been determined, and thus the comparisons drawn are of a semiquantitative character only. However, the differences in activity, visually noted, are clear. In each graph the values of the two controls are given, i. e., the viscosity of a 0.5-percent solution of thymonucleate in water shown by the continuous line in each chart, and the viscosity of the appropriately diluted fluids used. The viscosity values of the latter were used exactly in the calculation of the relative viscosities of the mixtures. Since, however, the comparatively small differences between the viscosity values for the fluids are not readily revealed in the graphs, a single averaged value is used for this control and is so represented by the broken line in each chart. At 16-cm. H_2O pressure, no changes in the values of the viscosities of the various fluids can be observed during the incubation interval. If any changes do occur in these values, they cannot be picked up at the high-velocity gradient used and hence do not affect the values observed for the much more viscous mixtures of the fluids with thymonucleate.

THE INTERACTION WITH THYMONUCLEATE

Pure Proteins, Amino Acids, and Salts

Greenstein and Jenrette (*5*) showed that proteins, glycine, and a wide variety of salts lower the structural viscosity and extent of streaming birefringence of thymonucleate. Of the salts, the most effective cation was guanidinium, the most effective anion was iodide. The data of this earlier study were obtained on fresh solutions within a short period after mixing. The time course of the interaction is discussed in the present study. In figures 1 and 2 are shown the values of the viscosities of mixtures of thymonucleate with pure

proteins, amino acids, and salts over 4 hours of incubation at 30° C. in the viscometers.

From the curves in figures 1 and 2, two conclusions may be drawn: (1) The various agents used lower the viscosity of the nucleate to an extent dependent on the nature and concentration of the agent; and (2) the rate of fall in the viscosity over the 4-hour period for the nucleate in water alone and in the mixtures is almost negligible.[2] Attention need, therefore, be only directed to the relative effect of the various agents used on lowering the viscosity values of the thymonucleate independently of the time of incubation. The remarkable effect of guanidine HCl, at a concentration as low as 10^{-5} M, reveals the properties of this salt (*5*, *7*). Sodium chloride at 0.5 M has a much weaker effect on the viscosity of thymonucleate than has guanidine HCl at 0.2 M. Arginine HCl and lysine HCl also are more effective than sodium chloride at equivalent concentrations. Arginine HCl differs from lysine HCl by the possession of a guanidine group substituted on the terminal carbon atom; the former lowers the viscosity of nucleate to a greater extent than the latter at equivalent concentrations, a result that might well be attributed to the presence of the guanidine group in the arginine molecule.

The purified proteins, crystalline egg albumin and horse-serum albumin, affect the viscosity of thymonucleate (fig. 2) to a remarkable degree if the concentrations

[2] *There is some evidence of a possibly significant drop in the viscosity with time of the mixture of nucleate with the most concentrated solution of egg albumin. The drop is not sufficiently great as to be a matter of concern at the present time, since it is barely more than that exhibited by the control.*

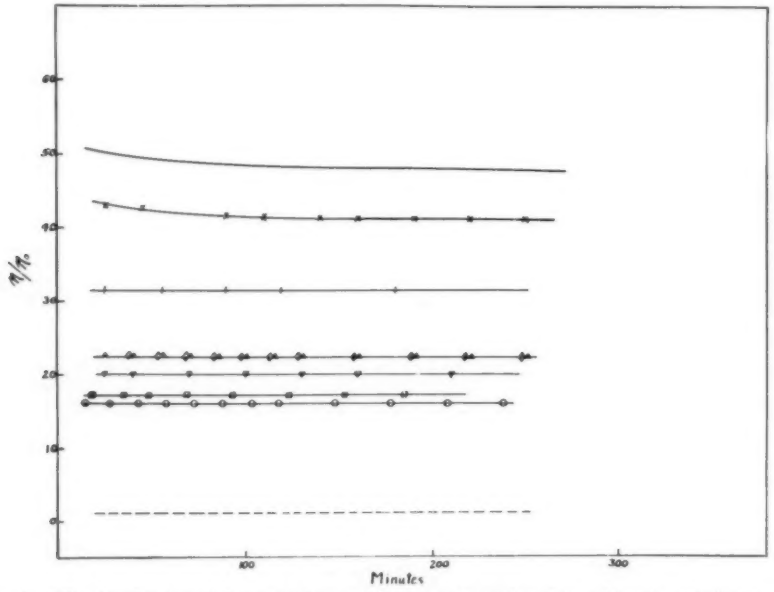

FIGURE 1.—The incubation of thymonucleate with inorganic and amino acid salts. Ordinate, relative viscosity: abscissa, time of incubation.

—— Control, ○ 2M Guanidine HCl, □ 0.2M Guanidine HCl, ◇ 0.002M Guanidine HCl, × 0.00002M Guanidine HCl, ▽ 0.5M Arginine HCl, △ 0.5M. Lysine HCl, l 0.5M NaCl, – – – – Control.

of protein used are placed on a molar basis. Thus, a solution of horse-serum albumin containing 2.46 mg. N per cubic centimeter lowers the relative viscosity of thymonucleate from about 50 to about 20. The concentration of protein in this solution is approximately 0.002 M. Yet the effect of lowering the viscosity of nucleate is very much greater than that of glycine at 1 M or histidine at 0.05 M. The same is true of the behavior of egg albumin. This property of proteins will be discussed in another section. For the present it need simply be pointed out that the effect of the proteins on lowering the viscosity of nucleate is proportional to the concentration of protein, and that at equal nitrogen concentrations egg albumin is more effective than serum albumin. Under such conditions the molar concentration of the former protein is about double that of the latter.

Histidine at 0.05 M concentration is more effective than glycine at 1 M, a phenomenon which may be ascribed to the presence of the imidazole ring in the former amino acid. However, glycine at as low a concentration as 1×10^{-4} M, nevertheless has a distinct effect in lowering the viscosity of thymonucleate.

In the case of every mixture, the streaming birefringence was distinctly positive although lower in degree than that of the control of thymonucleate in water.

Extracts of Tumors and Normal Control Tissues

The effect which tissue extracts exert on the physical properties of thymonucleate is illustrated in figures 3 and 4. Examination of the curves in these figures reveals that two distinct steps are involved in the interaction: (1) A decrease in the viscosity brought about by the presence of

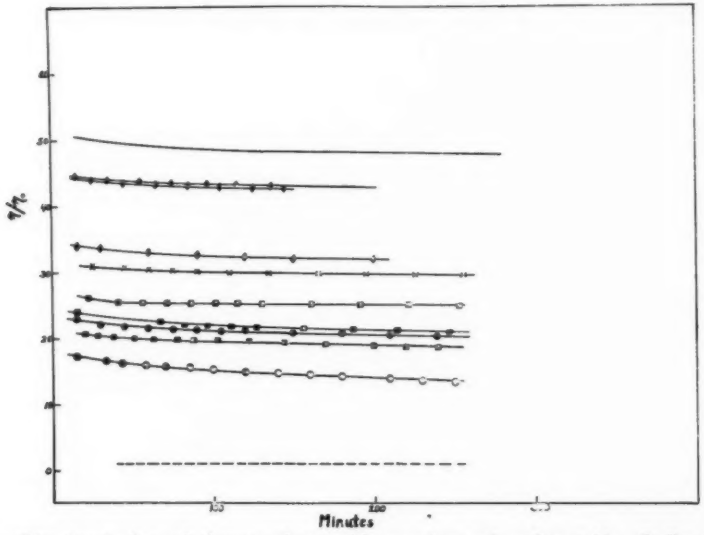

Figure 2.—The incubation of thymonucleate with proteins and amino acids. Ordinate, relative viscosity; abscissa, time of incubation.

—— Control, ● Egg Albumin, 2.46 mg. N per cubic centimeter, ⊗ 1.23 mg. N per cubic centimeter, ■ Serum Albumin 2.46 mg. N per cubic centimeter, ⊠ 1.23 mg. N per cubic centimeter, □ 0.41 mg. N per cubic centimeter, ◇ Glycine 1.23 mg. N per cubic centimeter, ◆ 1M Glycine, ⊗ 0.0001 M Glycine, × Histidine 0.05M, – – – – – Control.

the proteins and salts in the extract, a decrease which occurs almost at once after mixing; and (2) a very rapid rate of decrease within the incubation period, so rapid with certain extracts as to reduce the viscosity of the mixture to the value characteristic of the extract itself within a short space of time. The first step is simply that characteristic of proteins, amino acids, and salts as shown in figures 1 and 2. It is suggested in figure 3, for example, by the fact that the initial decrease (up to the first 15 minutes) is from a relative viscosity of about 50 to about 18. After this initial measured period, the rate of decrease is comparatively much slower, the viscosity dropping from about 18 to 5 in 4 hours. The first step is likewise suggested in figure 4 by the curves for the lactating and hyperplastic mammary tissues, which illustrate the great initial drop in viscosity soon after mixing, followed by the relatively slower decrease during the incubation period. Although the latter decrease in viscosity, illustrated in the curves, is lower in absolute amount than that shown by the initial drop, it is, nevertheless, a phenomenon which pure proteins, amino acids, and salts do not exhibit. The effect of these latter substances on the viscosity of thymonucleate after the initial drop is practically negligible. Furthermore, in many of the tissue extracts, the initial drop (within 15 minutes after mixing) is very much greater than that shown by an equivalent amount of pure protein. This fact is particularly illustrated by the case of the more concentrated extract of mammary tumor in figure 4.

The conclusion appears inescapable, therefore, that there must exist some factor in the tissue extracts which is not inert protein, amino acid, or salt, and which in

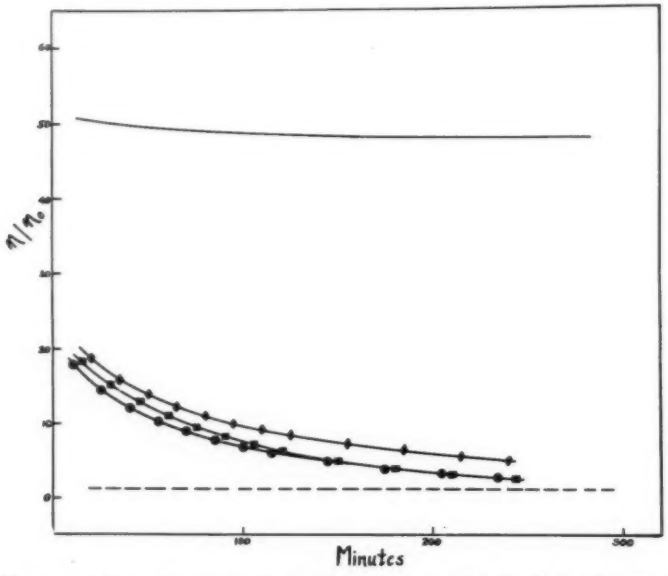

FIGURE 3.—The incubation of thymonucleate with rat liver extracts. Ordinate, relative viscosity; abscissa, time of incubation. Each extract contains 1.23 mg. N per cubic centimeter. —— Control, ■ Normal liver, ● Regenerating liver, ♦ Hepatic tumor, – – – – Control.

sufficient amount appears to be capable of reducing the viscosity of thymonucleate to that almost characteristic of water. That this factor causes the disintegration of the asymmetric molecules of thymonucleate is shown by the fact that when a sufficiently low value of the viscosity of the mixture is reached the streaming birefringence disappears. There is a distinct correlation between these two properties, and later in this communication we show that at the end of the incubation periods of mixtures of thymonucleate with certain tissues, when the viscosity of each mixture has become constant, the structural character of the viscosity disappeared. These phenomena can be interpreted on the basis that a complete, or nearly complete, depolymerization of the thymonucleate to the tetranucleotide stage has occurred, and that the agent responsible for this is an enzyme or system of enzymes exsisting in the tissues studied. Thus, when a solution of thymonucleate is treated with an appropriate tissue extract, two steps recognizably occur: (1) An initial drop in the viscosity and streaming birefringence due now not only to the presence of inert protein, amino acids, and salts but also to the presence of a depolymerizing factor in the extract; and (2) a gradual but marked decrease in these properties until a minimum value is reached. The activity of the enzyme factor superimposed upon the initial drop owing to the presence of proteins, etc., is responsible for the greater magnitude of the initial drop (1) in the case of tissue extracts than that shown in the case of solutions of pure proteins of equal nitrogen concentration.

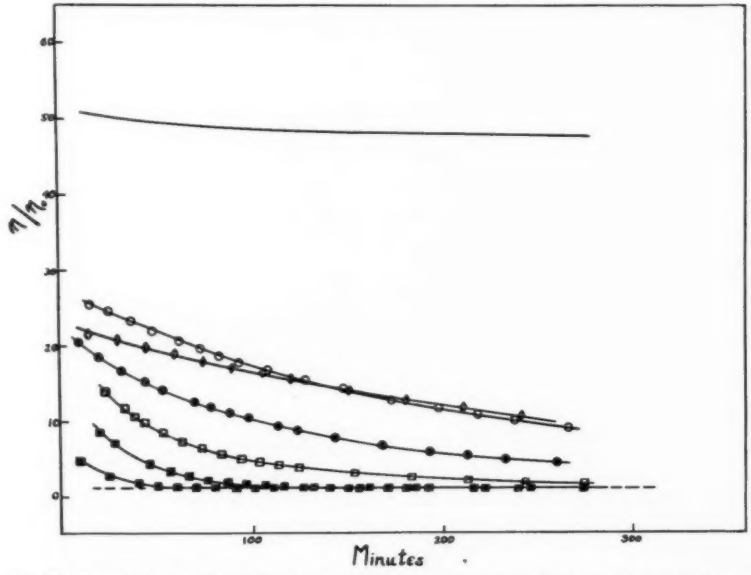

FIGURE 4.—The incubation of thymonucleate with mouse mammary tissue extracts. Ordinate, relative viscosity; abscissa, time of incubation.

—— Control, ○ Lactating breast 0.41 mg. N per cubic centimeter, ● Lactating breast 1.23 mg. N per cubic centimeter, ◇ Hyperplastic breast 0.41 mg. N per cubic centimeter, □ Mammary tumor (no necrosis) 0.41 mg. N per cubic centimeter, ■ Mammary tumor 1.23 mg. N. per cubic centimeter, ⊠ Mammary tumor (including necrotic areas) 1.23 mg. N per cubic centimeter, – – – – – Control.

Further evidence in favor of the presence of an enzyme factor lies in the fact that increasing the concentration of the same tissue extract in the mixture results in increasing not only the magnitude of (1), the initial drop, but also the subsequent rate of drop (2). This phenomenon is illustrated in figure 4. Still further evidence for the presence of the enzyme factor is adduced from experiments with serum described below.

Because of the changes in physical properties of thymonucleate brought about by the enzyme factor in the tissue extracts studied, changes interpretable only on the basis of a depolymerization, we have chosen to name this enzyme *thymonucleodepolymerase*. The exact mechanism of the action of this enzyme is as yet unknown—it is recognizable only by the physical effects which it produces. It may consist of a single factor or a number of factors. No phosphatase activity is associated with its effects, at least under the conditions which are employed in this investigation. The relative amount of the depolymerase in any given extract of tissue can be estimated by the rate at which a standard solution of thymonucleate diminishes in viscosity and streaming birefringence.

The curves in figure 3 and figure 4 reveal the presence of the depolymerase in both hepatic and mammary tumors and in the normal tissues from which the tumors are derived. There are significant differences in the relative activity of the depolymerase in the various tissues. At equal concentrations of total nitrogen, the activity of the rat hepatic tumor is less than that of normal rat liver and regenerating rat liver, the activity of the latter two tissues appearing to be about equal after a short period (fig. 3).

Examination of figure 4 reveals the very remarkable activity of the depolymerase in the extracts of spontaneous mammary tumors in C3H mice. Particularly is this true of the whole tumor, e. g., including necrotic areas, described by the crossed-in squares in figure 4. Fifteen minutes after an extract of this tissue is mixed with thymonucleate, the streaming birefringence of the latter disappears, and 1 hour after the mixing the viscosity of the mixture reaches a constant value little more than that of water. This phenomenon has been repeated over and over again with various samples of C3H mammary tumors. When the necrotic areas are carefully removed, the extract of the remaining tissue is somewhat less than before, but still extremely active. Comparison with the activity of the control tissues, the lactating and the hyperplastic breast, reveals the far greater amount of enzyme in the tumors. Indeed, the activity of the mammary tumor extract which has a concentration of 0.41 mg. N. per cubic centimeter is very much greater than that of an extract of lactating breast of three times this nitrogen concentration. After about 2 hours of incubation the activity of lactating and of hyperplastic breast appears to be nearly the same.

In direction, if not in relative order of magnitude, the distribution of thymonucleodepolymerase in the tissues studied is similar to that of arginase. Greenstein, Jenrette, Mider, and White (*6*) showed that the arginase activity of hepatic tumors was less than that of normal and of regenerating liver, and that the activity of mammary tumors in mice was greater than that of lactating and of hyperplastic breast. The same relative distribution of the depolymerase was noted in the present study. Minor differences were apparent, e. g., the arginase activity of hepatic tumors was very much less than that of normal liver, whereas the depolymerase activity of the tumor, while less than that of the liver, was present in comparatively

large amount. On the other hand, it was shown that the arginase activity of normal and regenerating liver was the same, and the activity of lactating and hyperplastic breast was very nearly the same (*6*). From the results of the present study, it appears that these similarities likewise hold for the depolymerase activity of the respective tissues mentioned.

The Milk of Various Species

The presence of the depolymerase in normal mouse breast led us to inquire whether it might not be present in the milk of the mouse. Milk was drawn from many animals by an aspirator developed in this Institute. Investigation of mixtures with thymonucleate revealed the presence of the depolymerase in mouse milk (fig. 5). This rather surprising finding led us to investigate the milk of a number of different animals. Several samples of the milk of the human, mare, cow, and goat failed to indicate the presence of the depolymerase. The results on these samples of milk are depicted in figure 5, where the data resemble those obtained on solutions of pure proteins. Thus, there is the initial drop in viscosity due to the proteins of the milk followed by a nearly constant value for the viscosity during the whole of the incubation period.

In contrast with the results obtained on the milk of these species, the results with the milk of the rat, mouse, rabbit, and guinea pig all revealed the presence of the depolymerase (fig. 5). The activity of mouse and of guinea-pig milk was practically identical. The activity of rabbit milk was not very marked, but that of rat milk was by far the most powerful of all the samples investigated. In relative order of magnitude, the activity even of rat milk is not so great as that found in extracts of mammary tumor or normal liver. However, the fact that the presence of the depolymerase is so readily revealed in the milk of certain species indicates that its amount is not negligible. The enzyme is synthesized presumably in the mammary tissues and finds its way subsequently into the milk. Its presence in the milk of the rodents is in any case unexpected. Whether the depolymerase serves a special function in the milk, or whether the latter fluid is a vehicle whereby the young acquire a ready-made enzyme system for the first period of life, or whether the presence of the enzyme is purely adventitious, remain for further investigation.

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The Sera of Various Species

The finding of the depolymerase in the milk of the rodents led us to investigate the sera of a number of animals of different species. The results of viscometric determinations on mixtures of sera with thymonucleate under identical conditions are shown in figure 6.

Inspection of figure 6 reveals that again there is an initial drop in the viscosity of the mixtures brought about by the presence of proteins, etc., in the serum, and that this drop is followed either by a fairly constant value during the incubation period or else by a decreasing approach to a lower limiting value during this period. Sera which followed the former path were derived from the human, horse, cow, and rat, and exhibited little or no depolymerase activity. Sera which followed the latter path, presumably because of their depolymerase activity, were obtained from the dog, guinea pig, mouse, and rabbit. There appears to be something of a difference between the behavior of rat and beef serum on the one hand and that of human and horse serum on the other within the first two hours of incubation (fig. 6). It is possible that there is a very slight activity of the

enzyme in rat and beef serum, which is exhibited in the early part of the incubation period, and which then levels off to a negligible value in the later stages. No trace of activity at any stage is shown in the case of human or of horse serum.

Of the sera which clearly demonstrate depolymerase activity, that of the dog is the most effective. Within an hour of mixing the dog serum with thymonucleate, the streaming birefringence of the latter disappears and the viscosity of the mixture is nearly that of the diluted serum itself.

The dog serum was separated by the usual procedures with ammonium sulfate into the three general protein fractions, albumin, euglobulin, and pseudoglobulin, and each fraction was investigated under identical conditions for the presence of depolymerase activity. The results are illustrated in figure 7.

Although the euglobulin fraction of dog serum is the only one which shows significant depolymerase activity, it is in no way as active as the whole serum. It would seem as if a considerable proportion of the activity of the serum was lost during the fractionation procedure. Recombination of the various fractions failed to restore the activity characteristic of the whole serum. However, quite apart from the absolute magnitude of the activity, it is sufficiently interesting to note that qualitatively the activity is characteristic of one of the protein fractions of the serum.

The various sera which were studied were obtained separately in many cases from males and females. No significant sex differences in the results were obtained either in the case of human serum, which does not contain the enzyme, or in the case of rabbit serum, which contains the enzyme.

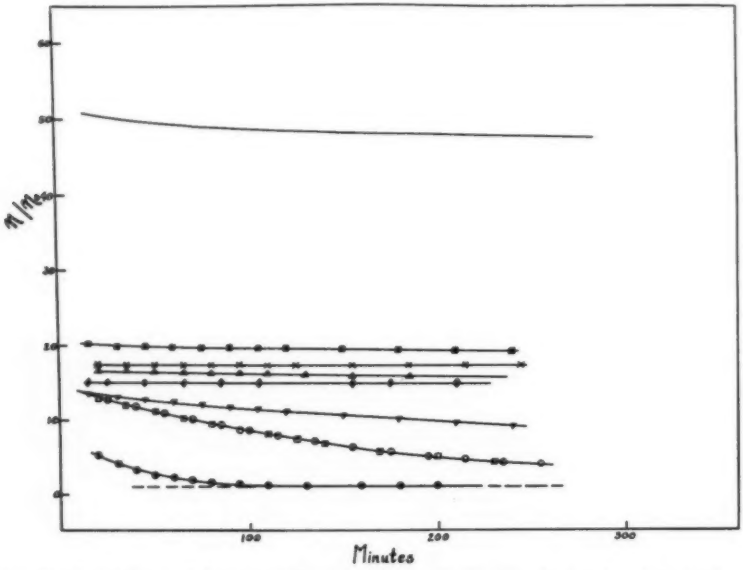

FIGURE 5.—The incubation of thymonucleate with milk. Ordinate, relative viscosity; abscissa, time of incubation. Each sample contains 2.46 mg. N per cubic centimeter.
—— Control, ○ Guinea pig, ◇ Goat, ● Rat, □ Mouse, ▽ Rabbit, △ Mare, × Cow, ■ Human, - - - - Control.

Attempts were made to see whether treatment of human and rabbit sera with oxidizing and with reducing agents would have any effect on the catalytic depolymerization of thymonucleate. These sera were treated, respectively, with neutralized cysteine and with an excess of porphyrindin, and allowed to stand for 1 hour before mixing with thymonucleate. The sera treated with cysteine stood in an evacuated Thunberg tube. The color of the added porphyrindin faded to a light orange during the period of standing. The results, however, on human and rabbit sera treated in this fashion were identical with those obtained on untreated sera.

In order to determine whether a dialyzable factor was present in the inactive human serum and in the active rabbit serum, samples of these sera were dialyzed against distilled water in the cold room at 5° C. for 2 weeks. The results on the dialyzed preparations, however, were identical with those obtained on undialyzed sera.

In order to determine whether there was any inhibiting factor to the depolymerase in an inactive serum such as that of the horse, the effect of a mixture of horse serum with active rabbit serum was investigated. A mixture of the two sera was prepared in which each was present to the concentration of 1.23 mg. N per cubic centimeter, the total N concentration being, therefore, 2.46 mg. per cubic centimeter. The activity of the mixture was then compared with that of horse serum alone (1.23 mg. N per cubic centimeter), and with that of rabbit serum alone (1.23 mg. N per cubic centimeter). The results are shown in figure

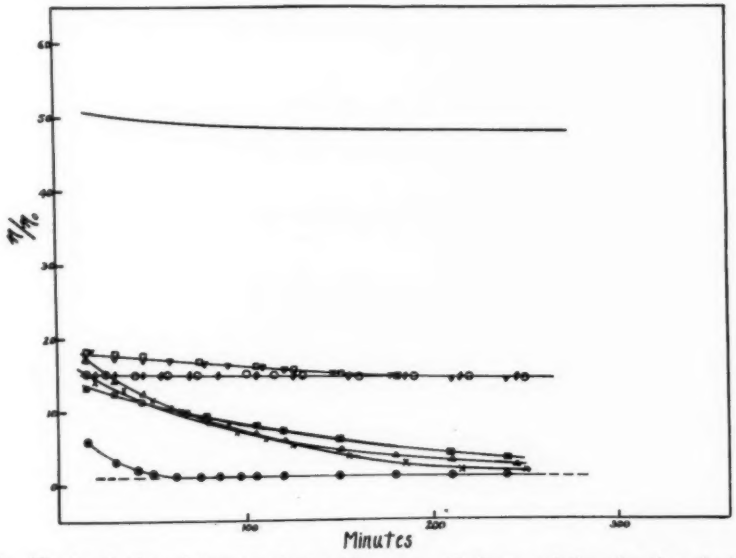

FIGURE 6.—The incubation of thymonucleate with serum. Ordinate, relative viscosity; abscissa, time of incubation. Each sample contains 2.46 mg. N per cubic centimeter.

—— Control, ● Dog, ◇ Human, ○ Horse, □ Beef, ▽ Rat, △ Guinea pig, ■ Mouse, × Rabbit, – – – – Control.

8. It is clear that the presence of the inactive horse serum had no other effect on the active rabbit serum in the mixture than to lower the viscosity value for the initial drop, a lowering which, of course, persisted throughout the entire incubation period during which the rabbit serum was effective. In other words, the mixture of the two sera, twice as concentrated in milligrams of N per cubic centimeter as either of the sera alone, began the incubation period at a more advanced depolymerization state than occurred with either serum alone. The degree of activity of the rabbit serum in the mixture and of the rabbit serum alone was practically identical, as is shown by the respective curves in figure 8, which are practically parallel.

Rat serum (fig. 6) has little or no depolymerase activity. On the other hand, rat milk (fig. 5) has the greatest activity of all the samples of milk investigated. In order to determine whether the serum of the rat during the lactation period might reveal some activity not observed in the nonlactating rat, parallel investigations on the sera of lactating and nonlactating rats were conducted. The results obtained in the two cases were practically identical. As a corollary to this investigation, a study was made of the sera of normal C3H mice and of the sera of C3H mice with spontaneous mammary tumors. No difference whatever could be observed in the degree of activity of depolymerase in the two samples of sera.

The Effect of Preheating Proteins and Sera Before Mixing With Thymonucleate

So far, it has been shown that oxidizing and reducing agents, dialysis, and the mixing of various sera have no observable

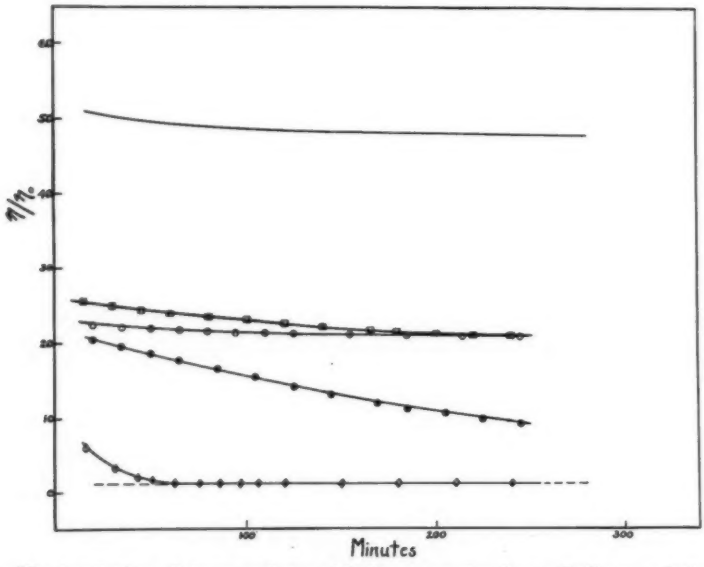

Figure 7.—The incubation of thymonucleate with dog serum fractions. Ordinate, relative viscosity; abscissa, time of incubation.

—— Control, ◇ Whole serum, ○ Albumin, □ Pseudoglobulin, ● Euglobulin, – – – – Control.

effect on the activity or nonactivity of specific sera. The number of agents which might be used for this purpose are limited, owing to the fact that materials which might affect the sera would also, unless conveniently removed beforehand, affect the nucleate after mixing. In addition, the limitations imposed by the use of the capillary viscosity method made it necessary to avoid any procedure which might lead to a heterogeneous mixture. However, sera and solutions of serum albumin will remain homogeneous after prolonged heating at 60°–63°, and it was determined to investigate the effect of preheating sera before mixing with thymonucleate.

When rabbit serum was heated at 60°–63° for 5 hours, then cooled and mixed with thymonucleate, it lost the depolymerase activity although it still retained the effect of causing the initial drop in the viscosity of the mixture. This is clearly revealed in figure 9. When, however, the rabbit serum was heated at this temperature for 15 hours, not only was the depolymerase activity gone, but it had lost most of its effect in causing the initial drop in viscosity (fig. 9). Heating of the serum for 40 hours before mixing with thymonucleate had very little more effect than that elicited by the 15-hour period. Horse serum preheated at 60°–63° for 5 hours before mixing with nucleate lost some of its ability to cause a drop in the viscosity of the mixture; after 15 hours at this temperature this ability had nearly disappeared (fig. 9). In all cases, the protein content of the sera was the same after heating as before (amount precipitated by 5 percent trichloracetic acid).

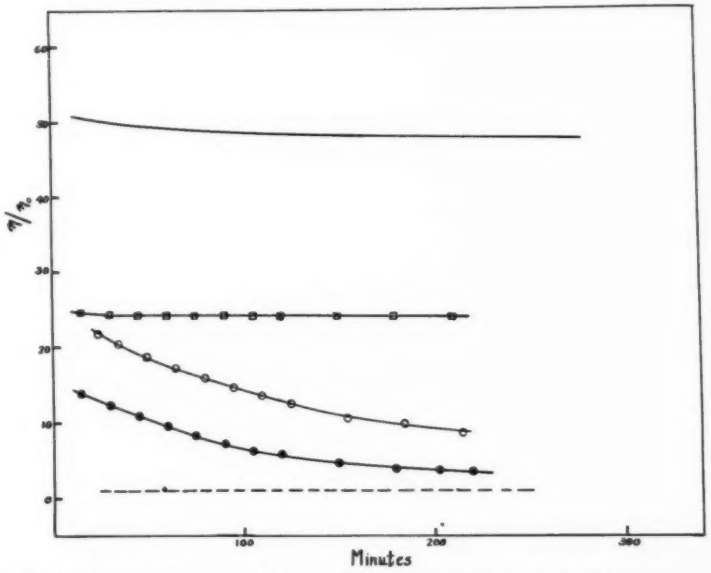

FIGURE 8.—The incubation of thymonucleate with a mixture of horse and rabbit serum. Ordinate, relative viscosity; abscissa, time of incubation.

—— Control, ○ Rabbit 1.23 mg. N per cubic centimeter, □ Horse 1.23 mg. N per cubic centimeter, ● Mixture containing 1.23 mg. N per cubic centimeter of each component.

The results obtained with heated sera are highly significant. Apart from the fact that they suggest that the depolymerase activity is destroyed by heat, they reveal that the ability of the proteins of the sera to lower the viscosity of thymonucleate is dependent upon the native unaltered state of these proteins. When the proteins are denatured by the prolonged standing at 60°–63°, the viscosity-lowering capacity, i. e., the depolymerizing capacity, is nearly destroyed. This capacity of the serum proteins is apparently a function of the native structure of the proteins and is not, primarily at least, a function of the kind of chemical groupings in the protein. These groupings, after all, are present both in heated and unheated sera.

A similar series of experiments with horse-serum albumin revealed that a much longer period of heating solutions of this purified protein is necessary to destroy the capacity for viscosity lowering than is necessary for the whole serum. In order to attain the same results shown by serum at 15 hours, it was necessary to heat the albumin solution for about 60 hours. This suggests that the complex mixture of proteins in the serum is more labile toward heat than is a single protein. Egg albumin could not be studied under these conditions because it coagulates quickly.

These results have an important bearing on the behavior of nucleoproteins *in vitro*. They strongly suggest that nucleic acid will not be affected by, or combine with a denatured protein, although it will be greatly affected by combination with any native protein. On the other hand, we may imply from these results that when the

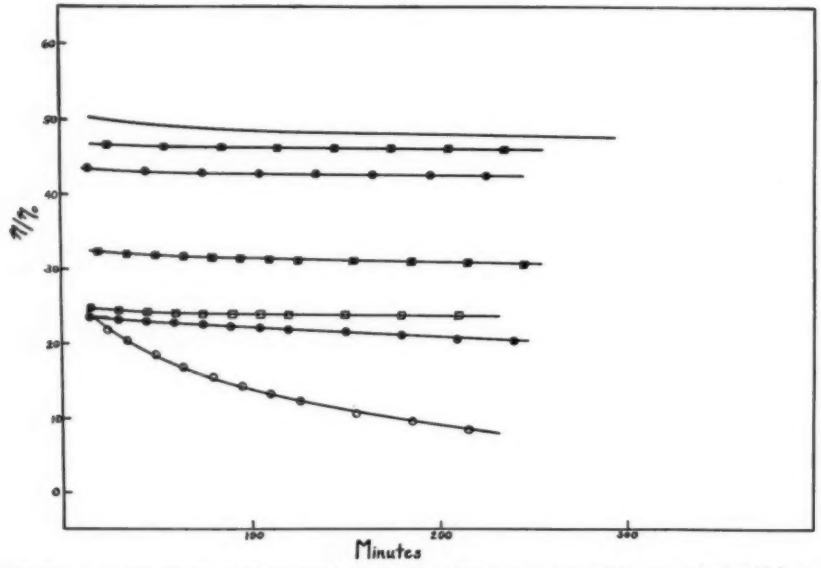

FIGURE 9.—The incubation of thymonucleate with preheated samples of horse and of rabbit serum. Ordinate, relative viscosity; abscissa, time of incubation. Each sample contains 1.23 mg. N per cubic centimeter.

—— Control, ○ Rabbit (unheated), ⊗ Rabbit (preheated 5 hrs.), ● Rabbit (preheated 15 hrs.), □ Horse (unheated), ⊠ Horse (preheated 5 hrs.), ■ Horse (preheated 15 hrs.), – – – – Control.

protein part of a nucleoprotein is denatured the nucleic acid will be released. This implication is supported by all experience on the isolation of nucleic acid from tissues, for it has invariably been necessary to denature completely all the proteins of the tissues in order to isolate the nucleic acid in the native, unaltered state. What this further implies is that the depolymerization of nucleic acid, effected by the native protein, is reversed when the protein is denatured. The evidence for this assumption may be illustrated by the results of the present authors (*11*) on the nucleoprotein of liver. This conjugated protein does not exhibit double refraction of flow or structural viscosity. When it is denatured, the nucleic acid split from it is highly viscous and doubly refracting during flow. The present authors have also shown that thymonucleate depolymerized to such an extent by salt that the streaming birefringence and structural viscosity have disappeared, will have these properties almost completely restored after physical removal of the salt (*5*). It is possible to speculate, therefore, on the rapid formation and breakdown of nucleoproteins induced by alternating and reversible native and denatured states of the tissue proteins concerned. However, further experimental work is needed on this.

The Structural Viscosity of Certain Mixtures After Attainment of Constant Viscosity

Although the various agents (extracts, proteins, etc.) lowered the viscosity of mixtures with thymonucleate, the determination of the viscosity was performed at a single pressure. In order to ascertain whether the structural character of the viscosity had likewise diminished or had been altogether destroyed, it was necessary to determine the viscosity at different pressures.

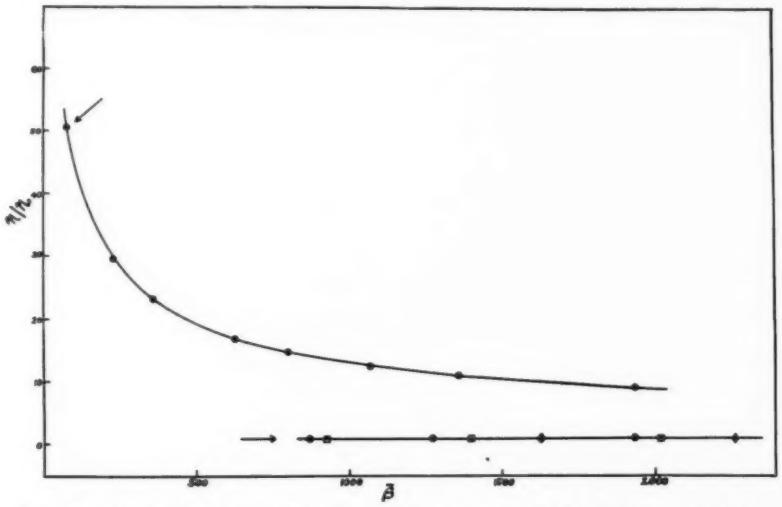

Figure 10.—The viscosity of mixtures of thymonucleate with various fluids as a function of the velocity gradient. Ordinate, relative viscosity; abscissa, relative gradient. The arrows indicate data at 16 cm. water pressure.

● Control, ○ Rat milk, ◇ Rabbit serum, □ Mammary tumor.

When the viscosity value at 16 cm. H_2O had reached a constant level for mixtures with thymonucleate of rat milk, rabbit serum, and mammary-tumor extract, the pressures were varied and the viscosities were determined at the new pressures for each mixture. Since the viscosity of these mixtures is little more than that of water, the range of pressures available is rather limited, but it is clear from figure 10 that the viscosities are independent of the velocity gradient employed. The mixtures had, therefore, lost the property of anomalous viscosity, a property originally belonging to the thymonucleate component of the mixture (control in fig. 10). The streaming birefringence also characteristic of the nucleate component had also disappeared. The thymonucleate at this stage may, therefore, be said to be completely, or nearly completely, depolymerized.

SUMMARY AND CONCLUSIONS

The presence of an enzyme in certain tissues, and in the milk and sera of certain species, which catalyzes the depolymerization of sodium thymonucleate, has been established.

The action of this enzyme, which we have named "thymonucleodepolymerase," has been followed by its effect on the viscosity and the streaming birefringence of thymonucleate mixed with the enzyme. During the incubation period of the mixture, these specific properties of the nucleate diminished steadily, the rate of diminution being considered proportional to the degree of enzymic activity in the tissue studied. When the viscosity in certain cases reached a lower, constant level, barely more than that of water itself, determinations at varying pressures indicated that the originally structural character of the viscosity had disappeared. The loss of these physical properties represents the criteria used for the depolymerization. No change in the inorganic phosphorus occurred during the incubation period.

The tissues studied comprised the following pairs of tumor and normal control tissues: (1) Transplanted hepatic tumor in rats and normal and regenerating rat livers; (2) spontaneous mammary tumors in C3H mice and lactating and hyperplastic mammary tissues, the latter induced by stilbestrol. Each of these tissues showed the presence of the enzyme. When the tissues were compared on the basis of an equal amount of nitrogen in the extracts, the results revealed that the mammary tumors were much more active than either lactating or hyperplastic mammary tissues. On the other hand, the hepatic tumor showed a lower activity than either normal or regenerating liver. Comparison of these findings with those on the distribution of arginase in normal and in cancerous tissues has been discussed.

The depolymerase is present in the milk of the rat, mouse, rabbit, and guinea pig. It is apparently absent in the milk of the human, cow, goat, and mare. When compared under similar conditions, the rat milk was most active of all the samples tested.

The depolymerase is present in the serum of the mouse, rabbit, dog, and guinea pig. It is apparently absent in the serum of the human and the horse. It may be present in a small trace in beef and in rat serum. Of the active sera studied, that of the dog was most effective. Fractionation of dog serum revealed that the activity is associated with the euglobulin fraction.

The effect on the viscosity and streaming birefringence of thymonucleate by admixture with tissue extracts, milk, and sera is revealed in two separate phenomena:

(1) An initial drop in these properties directly after mixing due to the effect of the proteins, amino acids, salts, etc.; and (2) either a maintenance of the values of these properties during the incubation period or else a gradual diminution in these properties. The inactive sera and samples of milk follow (1) and the former alternative of (2). The tissue extracts, and active sera and samples of milk follow (1) and the latter alternative of (2). When active rabbit serum is heated for 5 hours at 60°–63°, the depolymerase is destroyed but phenomenon (1) is maintained. When it is heated for 15 hours, not only (2) but also (1) very nearly disappears. The same holds true for the inactive horse serum. Denaturation of the serum proteins, therefore, destroys their ability to interact with thymonucleate. The bearing of these results on the theory of nucleoprotein formation is discussed.

REFERENCES

(*1*) LEVENE, P. A., and BASS, L. W.: Nucleic acids. New York (1931).

(*2*) FEULGEN, R.: Über a- und b-Thymonucleinsäure und das die a-form in die b-form überfuhrende Ferment (Nucleogelase). Ztschr. f. physiol. Chem., 237: 261 (1935).

(*3*) HAMMARSTEN, E.: Zur Kenntnis der biologischen Bedeutung der Nucleinsäureverbindungen. Biochem. Ztschr., 144: 383 (1924).

(*4*) SCHMIDT, G., PICKELS, E. G., and LEVENE, P. A.: Enzymatic dephosphorylation of desoxyribonucleic acids of various degrees of polymerization. J. Biol. Chem., 127: 251 (1939).

(*5*) GREENSTEIN, J. P., with the assistance of JENRETTE, W. V.: Chemical studies on the components of normal and neoplastic tissues. I. Viscosity and streaming birefringence of sodium thymonucleate. J. Nat. Cancer Inst., 1: 77 (1940).

(*6*) GREENSTEIN, J. P., JENRETTE, W. V., MIDER, G. B., and WHITE, J.: Chemical studies on the components of normal and neoplastic tissues. V. The relative arginase activity of certain tumors and normal control tissues. J. Nat. Cancer Inst., 1 687 (1941).

(*7*) EDSALL, J. T., and MEHL, J. W.: The effect of denaturing agents on myosin. II. Viscosity and double refraction of flow. J. Biol. Chem., 133: 409 (1940).

(*8*) ASTBURY, W. T., and BELL, F. O.: Some recent developments in the X-ray study of proteins and related structures. Cold Spring Harbor Symposia on Quantitative Biol., 6: 109 (1938).

(*9*) HAMMARSTEN, E.: Edward Gamaliel Janeway lecture; function of thymonucleic acid in living cells. J. Mt. Sinai Hosp., 6: 115 (1939).

(*10*) MAVER, M. E., and VOEGTLIN, C.: The chemistry of cell division. V. The influence of ascorbic acid, glutathione, and cysteine on the activity of tumor nuclease. Am. J. Cancer, 25: 780 (1935).

(*11*) GREENSTEIN, J. P., with the assistance of JENRETTE, W. V.: Chemical studies on the components of normal and neoplastic tissues. II. The nucleoprotein fraction of normal animal liver. J. Nat. Cancer Inst., 1: 91 (1940).

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